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NOTE CONCERNING THE IDENTITY OF VOLEMITOL AND α -SEDOHEPTITOL.

By F. B. LA FORGE AND C. S. HUDSON.

(Received for publication, June 18, 1928.)

Through the courtesy of Dr. F. Richter, editor of the Beilstein Supplements, it has been brought to our attention that an erroneous reference was given in our article on sedoheptose.¹ It was there stated that "the melting point of the benzal derivative of volemite is given by Bougault and Allard² as 90°, which does not agree with the melting point (200°) for the benzal compound of the heptitol [α -sedoheptitol]." The fact is, however, that Bougault and Allard did not describe such a compound of volemitol. It had been previously mentioned by Bourquelot³ as a substance crystallizing in silky needles (no melting point stated), and he also described a crystalline ethyl acetal of volemitol melting at 190°. Von Lippmann's "Chemie der Zuckerarten"⁴ recorded a m.p. of 90° for the benzal derivative of volemitol but it is quite apparent now that there is no authority for this statement and that it was erroneously repeated in our article. The data concerning volemitol and its derivatives that are recorded in several standard compilations contain so many erroneous statements that we have thought it advisable to record briefly here a correct record of the experimental data that have been published on the subject.

Volemitol was discovered by Bourquelot⁵ as a constituent of the rather rare mushroom *Lactarius volemus* Fr., m.p. 140–141° and $[\alpha]_D = +1.99^\circ$ or $+2.40^\circ$ in aqueous solution. This rotation is

¹ La Forge, F. B., and Hudson, C. S., *J. Biol. Chem.*, 1917, **xxx**, 61.

² Bougault, J., and Allard, G., *Compt. rend. Acad.*, 1902, **cxxxv**, 796.

³ Bourquelot, E., *J. pharm. et chim.*, 1895, **ii**, 389.

⁴ von Lippmann, E. O., *Chemie der Zuckerarten*, Brunswick, 1904, 2nd edition, 1002.

⁵ Bourquelot, E., *Bull. soc. mycologique France*, 1889, **v**, 132–163.

not affected by the addition of boric acid. In a later article Bourquelot⁶ records that the addition of borax greatly augments the dextrorotation of volemitol, $[\alpha]_D$ being about $+22^\circ$ to $+29^\circ$ in an aqueous solution containing 1.85 gm. of borax per 25 cc. A crystalline acetate of volemitol (m.p. 119° , $[\alpha]_D = +19.15^\circ$ in acetic acid), a crystalline ethyl acetal (m.p. 190° , levorotatory) and a crystalline benzal derivative (m.p. not recorded) are mentioned.

Fischer,⁷ working with a 10 gm. sample of volemitol which Bourquelot sent him, found its m.p. 149 – 151° after four recrystallizations from alcohol and its $[\alpha]_D^{20} = +1.92^\circ$ in 10 per cent aqueous solution. The oxidation of volemitol by bromine in alkaline solution yielded a reducing sugar which formed a crystalline phenylosazone and the analysis of this substance showed volemitol to be a heptitol.

Bougault and Allard² isolated crystalline volemitol from the roots and rhizomes of several species of *Primula* (*Primula grandiflora*, *elatior* Jacq. and *Primula officinalis* Jacq.) with a yield of about 15 gm. per kilo of dried plant. M.p. 154 – 155° , $[\alpha]_D = +2.65^\circ$ in aqueous solution. Boric acid did not affect the rotation, but borax increased it to $+20.83^\circ$ in about 7 per cent borax solution. The ethyl acetal melted at 206° and showed $[\alpha]_D = -46^\circ$ in chloroform solution. An acetate with a m.p. of 62° is mentioned but it is not clearly indicated whether the substance is crystalline. They conclude, however, that the acetate of m.p. 119° which Bourquelot described is not volemitol acetate but rather mannitol acetate and consider that Bourquelot's volemitol contained a small quantity of mannitol.

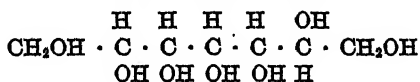
La Forge and Hudson¹ obtained α -sedoheptitol by the reduction of sedoheptose with sodium amalgam, m.p. 151 – 152° , $[\alpha]_D^{20} = +2.25^\circ$ in aqueous solution, and $[\alpha]_D^{20} = +22.09^\circ$ in about 6 per cent borax solution. The crystalline tribenzal derivative melted at 199 – 200° , but in a later article⁸ this value was corrected to 225° .

⁶ Bourquelot, E., *J. pharm. et chim.*, 1895, ii, 385–390. In this article Bourquelot refers to a publication by himself, "Sur la volémité, nouvelle matière sucrée retirée d'un champignon" in the *Procès-verbaux de l'association française pour l'avancement des sciences*, session de Pau, 1892, 183. This short publication is a summary of work more fully described in Bourquelot's other articles.

⁷ Fischer, E., *Ber. chem. Ges.*, 1895, xxviii, 1973.

⁸ La Forge, F. B., *J. Biol. Chem.*, 1920, xlii, 375.

La Forge⁸ recrystallized a sample of volemitol furnished him by Bourquelot and found a m.p. of 151°, and this value was not changed when the material was mixed with α -sedoheptitol. He prepared the ethyl acetal of α -sedoheptitol and found a m.p. of 191–194° and $[\alpha]_D = -45^\circ$ in chloroform solution. The agreement of these data with the results of Bourquelot (m.p. 190°) and of Bougault and Allard (m.p. 206°, $[\alpha]_D = -46^\circ$) led him to conclude that volemitol and α -sedoheptitol are identical, and that the configurational formula of volemitol is in consequence that which he had proved for α -sedoheptitol; namely,



or its mirror image.

Finally, the data which show the identity of α -sedoheptitol with volemitol are here summarized.

	Volemitol.		α -Sedoheptitol.	
	m.p.	$[\alpha]_D$	m.p.	$[\alpha]_D$
	°C.	degrees	°C.	degrees
Alcohol.	151	+2.65 (Water.) +20.8 (Borax solution.)	151	+2.25 (Water.) +22.1 (Borax solution.)
Ethyl acetal.	(206) (190)	-46 (CHCl ₃)	191– 194	-45 (CHCl ₃)

The alcohols when mixed show a m.p. of 151°.

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THE PRECIPITATION OF BLOOD CALCIUM BY LEAD.

BY FRITZ BISCHOFF AND L. C. MAXWELL.

(From the Chemical Laboratory of the Potter Metabolic Clinic and the Department of Cancer Research, Santa Barbara Cottage Hospital, Santa Barbara.)

(Received for publication, June 19, 1928.)

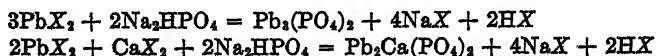
INTRODUCTION.

The treatment of inoperable cancer by the intravenous injection of suspensions of lead has stimulated interest in the fate of this metal in the blood stream. The original metallic colloid of Bell (1) is very sensitive to oxygen, and by *in vitro* experiments Brooks (2) and Bischoff and Blatherwick (3) showed that there was little likelihood of any of the metallic lead reaching the cancer as such. Aub and Reznikoff (4) had shown that the phosphates and carbonates were the only two of nine constituents normally found in blood which would neutralize the damaging effect of ionic lead to red cells, the phosphate ions being many times as effective as the carbonate. Fairhall and Shaw (5) found that the lead deposited in bones in lead poisoning is present as phosphate and not as carbonate or albuminate, and from a critical study of the solubilities of lead carbonate, dilead phosphate, and trilead phosphate, concluded that trilead phosphate was the form in which lead was transported in the blood in lead poisoning. Bischoff (3, 6) prepared various colloidal suspensions of lead with the inorganic constituents of the blood and found from rabbit tests that colloidal metallic lead, ionic lead and lead hydroxide were about equally damaging to the red blood cells. The oxycarbonate was less damaging and the phosphate without effect. Ionic lead, buffered with serum or red blood cells containing sufficient inorganic phosphorus to convert all the lead to phosphate, was also found to be non-toxic. The colloidal phosphate prepared for the rabbit experiments was then tried clinically by Ullmann (7), Pulford (8), Soiland, Costolow, and Meland (9), and others.

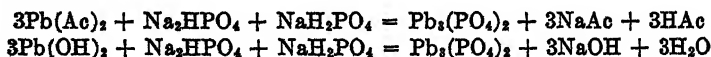
Brooks (2), experimenting with the Bell metallic colloid and ionic lead, found that when these substances were added to serum, the amount of phosphorus precipitated from the serum (determined by analyses of ultrafiltrates), was higher than the theoretical amount required to convert the lead to the triphosphate and corresponded more nearly to the diphosphate. Brooks explained his results by assuming a delayed equilibrium in which the dilead phosphate was first formed. At the end of 70 hours, the ratio of lead to phosphorus still corresponded to the dilead phosphate. Bischoff

6. Calcium Precipitation by Lead

(6) showed that when ionic lead is added to the blood, the amount circulating after 2 hours is nil. On the basis of Brooks' interpretation, there would be little chance of any trilead phosphate being formed from the metallic colloid by the time the lead reached the tumor, and the experimental evidence for the clinical use of the trilead phosphate would be materially weakened. From his lead:phosphorus ratios, Brooks (2) concluded that in a phosphate buffer, lead acetate and colloidal metallic lead formed the triphosphate; in Ringer's solution the former formed the triphosphate and the latter the diphosphate, and in blood serum they both formed the diphosphate. These results suggested to us that calcium might be thrown out with the lead in the cases where the ratio pointed to the diphosphate, and that no dilead phosphate but a precipitation of lead and calcium triphosphates or a double triphosphate salt took place. Brooks' analytical results would be in complete harmony with this explanation.



His results with Ringer's solution made this explanation seem likely, since this solution becomes highly supersaturated with calcium when the pH is alkaline. In the case of lead acetate in which he obtained the ratio for the tri-salt the Ringer's solution would become more acid. For the colloidal lead, where the ratio for the di-salt was obtained, it would become more alkaline.



Object.

The experiments recorded in this paper were taken up with the idea of establishing whether or not calcium was involved in the reaction of lead ions with blood serum. A few preliminary experiments showed that ultrafiltrable calcium was thrown out of solution. The amounts of phosphorus and calcium removed from solution were greater than is required for the above equations. In order to interpret these results in the light of recent investigations as to the state of calcium in the blood, complete data for pH, CO₂-combining power, inorganic phosphorus, and diffusible and non-diffusible calcium were taken.

Analytical Methods.

Inorganic phosphorus was determined by the Fiske-Subbarow modification of the methods of Bell and Doisy and Briggs. The maximum experimental error was within 5 per cent. Calcium

was determined by the Clark-Collip modification of the Kramer-Tisdall method. Duplicate analyses checked well within 0.3 mg. of Ca. CO_2 -combining power was determined by the Van Slyke gasometric method and in some experiments by the manometric method. Analyses of our ultrafiltrates showed a variation of 5 per cent in CO_2 -combining power as compared with the value obtained for the original serums. The pH was determined colorimetrically with Clark and Lubs' indicators and standard buffer solutions. Values were duplicated within 0.05 pH in the range of pH 6.8 to 7.4 and within 0.1 pH for the other values given.

Experiments with Ringer's Solution and Phosphate Buffer.

The Ringer's solution contained 8 gm. of NaCl, 0.2 gm. of KCl, 0.22 gm. of CaCl_2 , 2.22 gm. of NaHCO_3 , and 0.24 gm. of KH_2PO_4 in 900 cc. The pH was adjusted to 7.2 by adding dilute HCl. To 180 cc. of this solution were added 20 cc. of a mixture containing 42 mg. of PbCl_2 and 2.3 cc. of 0.1 N NaOH. The pH after the addition of the basic lead solution shifted to 8.2. The mixture was stirred half an hour and filtered. 6.2 mg. of Ca and 4.0 mg. of P per 100 cc. were found in the filtrates. The original Ca and P on a 100 cc. basis were 8.4 and 6.0 mg. respectively. The amount of phosphorus removed from solution lies between the values required for the di- and triphosphates. There is not enough phosphorus removed from solution to convert both the lead and calcium to the triphosphate. The results of the experiment in Ringer's solution indicated that some calcium carbonate was also thrown down with the phosphate. The experiment was tried in a phosphate buffer solution containing calcium chloride. It is interesting to note that both this phosphate solution and Ringer's containing calcium chloride were supersaturated with respect to calcium phosphate. Thus a calcium chloride-phosphate solution was made by adding 0.24 gm. of KH_2PO_4 , and 11 cc. of 0.1 N NaOH solution to 900 cc. of CO_2 -free water at 60°. To this solution 0.22 gm. of CaCl_2 in 100 cc. of water was added. The mixture was placed in a thermostat for 1 hour at 37° and a slight precipitate filtered off. The analysis of the filtrate gave 5.3 mg. of P and 5.0 mg. of Ca per 100 cc. The pH was 6.7. If the ionic strength is taken as 0.098 and the

apparent solubility product at that ionic strength as pK 31.0 from Holt, La Mer, and Chown's (10) data for the solubility product of tricalcium phosphate, at 37°:

$$\begin{aligned}(\text{Ca})^3 (\text{PO}_4^{\equiv})^2 &= (\text{solubility product}) \\ X^3 (1.71 \times 10^{-3}) (6.7 \times 10^{-7})^2 &= 1.0 \times 10^{-31}\end{aligned}$$

where 1.71×10^{-3} is the concentration of phosphorus in mols per liter, and 6.7×10^{-7} is the fraction present as PO_4^{\equiv} at the given pH.

$$\begin{aligned}X &= 9.2 \times 10^{-5}, \text{ mols Ca for saturation} \\ 1.25 \times 10^{-5}, &\text{ mols Ca present}\end{aligned}$$

The filtrates remained perfectly clear for days. To two 100 cc. portions of such a phosphate-calcium chloride solution were added 50 mg. of lead acetate and 50 mg. of lead acetate neutralized with NaOH, respectively. The original pH of the buffer solution was 7.3. Adding lead acetate changed the pH below 6.0. Adding the neutralized acetate took the pH to over 9.0. 2.9 mg. of P were removed in the lead acetate experiment (theory for the triphosphate 2.75 mg.). 4.4 mg. of P were removed in the basic lead experiment (see Table I). These experiments were performed at room temperature, 23°. The experiment with the phosphate buffer and lead acetate was repeated at 37° with the same results. An experiment with Ringer's solution under CO_2 tension was also performed at 37°. In the experiments with phosphate buffer a considerable shift in pH was brought about by the liberation of acetic acid, because the concentration of phosphate (about the same as in blood) was low. In Ringer's solution the relatively high concentration of bicarbonate lessened this effect. The results of this experiment were particularly interesting. An amount of phosphorus corresponding to trilead phosphate was thrown out of solution, confirming a similar experiment of Brooks; at the same time all the calcium down to a concentration of 0.2 mg. per 100 cc. was removed after standing 1 hour. After the solution had stood 30 hours the amount remaining was not titratable. In the analysis for calcium 5 times the volume of solution usually taken was used to increase the accuracy; this was necessary because of the small amounts of calcium remaining in solution. The experiment shows that at the pH of the

Effect of Lead Ions upon Ringer's Solution, Calcium Chloride-Phosphate Solution, and Blood Serum.

Medium.	Before lead.				After lead.					Phosphorus calculated.				Ca removed.
	pH	Inorganic P. mg. per l.	Total Ca. mg. per l.	Dialysable Ca. mg. per l.	Pb added. mg. per l.	pH	Inorganic P. mg. per l.	Combined CO ₂ mg. per l.	Dialysable Ca. mg. per l.	P removed. mg. per l.	Required for Pb ₃ (PO ₄) ₂ mg. per l.	Required for Pb ₃ (PO ₄) ₂ Ca ₃ (PO ₄) ₂ mg. per l.	Required for PbHPO ₄ CaHPO ₄ mg. per l.	
Ringer's solution + Pb(Ac) ₂	7.7	59	18.5	338	338	7.55	25	838	0.5	34	33.8	51.0	76.4	1.37
" + Pb(OH) ₂	7.2	60	84	156	156	8.2	39		62	21	15.6	27.0	40.5	∞
Phosphate-CaCl ₂ + Pb(Ac) ₂	7.3	54.5	79	275	275	<6.0	25.4		79	29	27.5			∞
" + ".....	6.7	53	50	275	275	4.9	27		50	26	27.5			1.33
" + Pb(OH) ₂	7.3	54.5	79	275	275	>9.0	6		(39)	48	27.5	(48.1)	(72.2)	1.33
Serum.														
Experiment I.....	8.3	67	140	96	268	8.3	9.5		57.5	577	26.8	46.7	70	1.34
" II.....	8.2	52	140	97	110	8.2	32	294	75	20	11.0	22.4	33.5	0.97
" III.....	7.35	47	161	108	159	7.15	14	670	80	33	15.9	30.4	45.5	1.09
" IV.....	7.3	71.5	145	105	198	7.25	36	520	70.4	35.5	19.8	37.7	47.5	1.09
" V.....	7.55	88	112	90	259	7.45	34.9	1022	43	53	25.9	50.2	75.2	1.07
Serum and oxalate.														
Experiment VI.....	80	12	8.6	520	520	7.1	28	700	1.3	52	48	52		
" VII.....	50	32	10.4	394	394	6.95	9	773	2.1	41	35.3	39		

The figures in parentheses indicate calculated values.

blood, lead acetate throws out both the calcium and the phosphorus, but that the amount of phosphorus is not sufficient for the triphosphate of both the lead and the calcium. Some carbonate is undoubtedly removed at the same time (see Table I). The data will be discussed again in connection with the experiments with blood serum.

These experiments showed that the difference in results obtained by Brooks in a phosphate buffer and in Ringer's solution could be accounted for by precipitation of calcium, and did not indicate the formation of lead diphosphate. It will be noted that basic lead suspensions, prepared just before use, were taken instead of metallic colloidal lead solutions. This was done because colloidal lead solutions always contain appreciable amounts of lead carbonate (10 per cent) and since it has been shown that the colloidal lead is rapidly oxidized to lead hydroxide when added to a solution saturated with air.

Experiments with Blood Serum.

The apparatus described by Brooks (2) was used in the experiments with blood serum. The collodion bags were made from du Pont parlodion dissolved in the amounts of alcohol-ether recommended for surgical collodion. Filtration was conducted under a pressure of 200 to 300 mm. of Hg. A known volume of serum was introduced into the bag, and a sufficient quantity ultrafiltered to determine the pH, calcium, phosphorus, and bicarbonate, and to make a biuret test. In the first three experiments the lead acetate peptized in 2 cc. of serum was added to the serum in the bag. After thorough mixing, the ultrafiltration was continued, the first few cc. being discarded. In the fourth experiment, the lead acetate was not added to the serum remaining in the bag, but to a fresh sample of the same serum. In the fifth experiment the lead acetate was added directly to the total volume remaining in the bag. It was thought that the way in which the lead was added might affect the results. Alveolar air was used in the CO₂ tension experiments. The apparatus was first swept out with this gas mixture, which was also bubbled through the serum several minutes before the serum was introduced into the bag. A stream of the mixture was also kept bubbling through the serum while the lead acetate was being peptized and mixed with the serum. In

the first four experiments rabbit serum was used; in the fifth pig serum. The ratio of diffusible to total calcium for the four rabbit serums was fairly constant, varying from 67 to 72.5 per cent. The total calcium value is about 3 mg. higher in the rabbit serum than the value given for human serum. There was sometimes a slight difference between the inorganic P and always a difference between the bicarbonate values for the serum and its ultrafiltrates. The values for the ultrafiltrates were used in the calculations. The serums were preserved with phenol. One serum was used without the addition of a preservative.

The preliminary experiments with blood serum were performed without CO_2 tension. The experiments with phosphate buffer containing calcium chloride had shown that the precipitation of the calcium depended upon the pH of the medium; lead acetate, which produced a greater acidity, throwing down no calcium. Brooks had shown that in serum there was no difference in the phosphorus removed from solutions whether one used lead acetate or colloidal lead. Our experiments were confined to lead acetate. From the solubility product theorem, the calcium in the serum should become more supersaturated as the alkalinity is increased. With use of lead acetate this condition would not arise.

The results of our experiments showed that calcium was thrown out of solution whether one worked at a pH above 8 without CO_2 tension or at a pH of 7.4 with CO_2 tension. The amount of calcium thrown out of solution was equivalent to the amount of lead added. The amount of phosphorus removed from solution corresponded to the triphosphates of lead and calcium. It will be noted that the phosphorus removed from solution is higher than most of the values reported by Brooks and that the calcium is twice as high as would be required if one interpreted Brooks' findings on the basis of a mixed tricalcium lead phosphate instead of dilead phosphate. Brooks gives two values which are 10 per cent, and one which is 5 per cent higher than the theoretical value of his diphosphate. His other values are 5 to 10 per cent lower. In determining the phosphate value, the difference between two analytical values which may be in error by 5 per cent, is taken. The same applies to the calcium, for while the analytical values for calcium are probably more accurate than those for phosphorus, the difference between the calcium values is a smaller per cent.

than the difference between the phosphate values. The higher values for phosphorus that we obtained in our experiments may be due to a difference in technique. The constancy of the ratio for added lead to precipitated calcium is well within the analytical error.

The amount of acid liberated by the reaction of the lead acetate with the phosphate in the serum is not great enough to shift the pH more than 0.2. Thus in Experiment III, the magnitude of the concentration of the various compounds expressed in mols per liter, was as follows:

7.65×10^{-4} lead added.	} Calculated from the M_2HPO_4 : MH_2PO_4 ratio at the initial pH.
6.45×10^{-4} acetic acid liberated.	
9.08×10^{-4} sodium acetate liberated.	
4.5×10^{-4} inorganic phosphorus left.	
1.65×10^{-3} bicarbonate.	

The acetic acid liberated is equivalent to but 5 per cent of the bicarbonate content.

TABLE II.

Apparent Solubility Products for $CaCO_3$ and $Ca_3(PO_4)_2$ in Serum after Lead.

The concentrations are expressed in mols per liter.

pH	$[Ca] \times 10^{-3}$	Total $CO_2 \times 10^{-3}$	$[CO_2] \times 10^{-3}$	P $\times 10^{-3}$	$[PO_4] \times 10^{-3}$	$[Ca] \times [CO_2] \times 10^{-6}$	pK $CaCO_3$	$[Ca]^3 \times [PO_4]^2 \times 10^{-25}$	pK $Ca_3(PO_4)_2$
8.3	1.43			0.36	23.2			15.7	23.8
8.2	1.88	0.67	20.8	1.03	61.8	39.0	6.41	236	22.6
7.15	2.00	1.5	3.23	0.45	1.51	6.46	7.19	0.182	25.7
7.25	1.76	1.27	3.38	1.16	5.39	5.47	7.23	1.58	24.8
7.45	1.08	2.43	10.4	1.13	9.9	11.2	6.95	1.23	24.9

Mechanism of the Calcium Precipitation.

The work of Holt, La Mer, and Chown and Hastings, Murray, and Sendroy (11) has shown that the amount of calcium in blood serum greatly exceeds the amount which should be present, calculated from the solubility product of calcium carbonate or calcium phosphate as determined in a salt mixture of the same ionic strength as serum. Whether this calcium is in a super-saturated, a colloidal, or a non-ionizable state does not appear to be definitely established. In order to interpret our data in the

light of the solubility product conception, the apparent solubility product for calcium carbonate and calcium phosphate has been calculated from the analytical data of our serum ultrafiltrates (see Table II). Since the calcium in these filtrates has passed through a membrane which holds back proteins, it is obviously not in a colloidal state nor bound in a non-diffusible form to protein. The CO_2 ion and PO_4 ion concentrations are calculated from the tables furnished by Hastings, Murray, and Sendroy (11) for carbonate, and Holt, La Mer, and Chown (10) for phosphate. In the calculation of Hastings, Murray, and Sendroy, the ionization constant is corrected for ionic strength. This was not done by Holt *et al.* Since our data are calculated for comparative and not absolute values, this difference is not essential to the interpretation of the results.

One might suppose that when calcium phosphate is thrown out of serum by the addition of lead acetate, an equilibrium would be established between the calcium phosphate in the solid and solution phases, and a constant solubility product would be obtained. Our data show that this is not the case (see Table II). The apparent solubility product for $\text{Ca}_3(\text{PO}_4)_2$ varies in each experiment. An error of 10 per cent in calcium and 0.1 in pH would affect the $\text{pK}_{s,p} \text{Ca}_3(\text{PO}_4)_2$ by 0.35 and the $\text{pK}_{s,p} \text{CaCO}_3$ by 0.16. In these experiments, the ultrafiltrates obtained 1 hour and 24 hours after the addition of lead were analyzed with no change in the results, so that equilibrium had apparently been reached. Since the solubility of calcium salts is not appreciably affected by the changes of temperature, we felt justified in comparing our solubility products, which were obtained at 23° , with those of Hastings, Murray, and Sendroy and Holt, La Mer, and Chown at 37° . Hastings, Murray, and Sendroy give pK CaCO_3 as 6.40 in serum and 7.40 in salt solutions of the same ionic strength. Our pK values at pH 7.15 to 8.3 for CaCO_3 equal or exceed 6.40, so that at equilibrium, the solution is not supersaturated with respect to calcium carbonate. Holt, La Mer, and Chown give 26.0 as the $\text{pK Ca}_3(\text{PO}_4)_2$ in serum and 27.2 in salt solutions of the same ionic strength. All our pK values are below this figure. The serums are still supersaturated with respect to calcium phosphate after the lead has been added. Sendroy and Hastings (12) have shown that when serum is shaken with solid CaCO_3 , no precipita-

tion of calcium takes place, but that when $\text{Ca}_3(\text{PO}_4)_2$ is used, the calcium content of the serum decreases rapidly, equilibrium being reached in 5 minutes. Our results would therefore tend to show that the calcium was not precipitated as $\text{Ca}_3(\text{PO}_4)_2$ but as a mixed lead-calcium phosphate. The constant ratio of lead added to calcium precipitated could better be explained on this basis. If the precipitation of this double lead-calcium phosphate is not governed by the solubility product for tricalcium phosphate, and if the double salt is more insoluble than calcium phosphate, the calcium should be precipitated from a solution which was not saturated with respect to calcium phosphate. In order to test this experimentally, over 90 per cent of the calcium was removed from a serum with sodium oxalate. The calcium remaining in solution did not exceed the amount required for saturation. Upon addition of lead acetate to this solution, all the calcium was precipitated.

EXPERIMENTAL.

Precipitation of Calcium beyond the Saturation Point of Tricalcium Phosphate.

It was found that when the amount of oxalate theoretically required to convert the calcium to oxalate was added to the serum, 10 per cent of the total calcium was not precipitated and a little less than 1 mg. per 100 cc. was still ultrafiltrable. Thus 14 mg. of sodium oxalate were added to 30 cc. of serum which analyzed 13 mg. of calcium per 100 cc. After the mixture had stood overnight the calcium oxalate was centrifuged off; 1.2 mg. per 100 cc. of calcium remained, of which 0.86 mg. per 100 cc. was ultrafiltrable. To 37 cc. of another serum were added 15.4 mg. of sodium oxalate. The total calcium was reduced from 13.2 mg. per 100 cc. to 3.2 mg. per 100 cc. The ultrafiltrable calcium was 1.04 mg. per 100 cc. Lead acetate was added to these serums under CO_2 tension, as in the other serum experiments, and the calcium determined in the ultrafiltrates. In order to increase the accuracy of the calcium determinations, double the volume usually taken was used in the analytical determination. All the calcium, within the limits of the determination, was thrown out by the lead.

If pK 26.0 is taken as the solubility product of calcium phosphate in serum,

$$X^3 ((0.94 \times 10^{-3})(2.8 \times 10^{-6}))^2 = 10^{-26}$$

$$X = 1.1 \times 10^{-3}$$

or 4.4 mg. of Ca per 100 cc. required for saturation in Experiment VI with serum.

$$X^3 ((0.29 \times 10^{-3})(1.7 \times 10^{-6}))^2 = 10^{-26}$$

$$X = 3.5 \times 10^{-3}$$

or 14 mg. of Ca per 100 cc. required for saturation in Experiment VII with serum.

In these calculations, 0.94×10^{-3} and 0.29×10^{-3} are the concentration of P in mols per liter after the addition of the lead, and 2.8×10^{-6} and 1.7×10^{-6} are the factors which give the amount of PO_4 ion at the pH of the solution. It will be noted (Table I) that in the experiments recorded above the phosphorus removed from solution corresponds very nearly to the amount required for trilead phosphate. In the calculations given in Table I, we have corrected the results for the amount of lead which would be precipitated as oxalate. This amount is small and does not materially affect the final result.

*An examination of the data for the experiment in which lead acetate was added to a Ringer's solution with a low calcium concentration, shows that the calcium has also been removed below the saturation point for either calcium carbonate or calcium phosphate. As this experiment was performed at 37° and the solid and liquid phases were in contact for 30 hours, a direct comparison with the data of Holt, La Mer, and Chown and Hastings, Murray, and Sendroy may be made. Thus:

$$X (1.9 \times 10^{-2}) (5.5 \times 10^{-3}) = 3.9 \times 10^{-8}$$

$$X = 3.8 \times 10^{-4} \text{ mols of Ca required for saturation of } \text{CaCO}_3.$$

$$X^3 ((8.1 \times 10^{-4}) (1 \times 10^{-5}))^2 = 6.3 \times 10^{-28}$$

$$X = 2.1 \times 10^{-4} \text{ mols of Ca required for saturation of } \text{Ca}_3(\text{PO}_4)_2.$$

These figures are equivalent to 1.52 and 0.84 mg. of Ca per 100 cc. of solution. The amounts found were 0.2 mg. after 1 hour and a trace after 30 hours. In this experiment there must have been some carbonate thrown down as well as phosphate. (The data

show that carbonate was also thrown down when Ringer's solution was treated with lead hydroxide.) The significant fact is that calcium may be precipitated by lead below the saturation point in salt solution as well as in serum. An analogous example is the precipitation of radium with barium. The interpretation of this phenomenon by Lind (13) in Taylor's "A Treatise on Physical Chemistry" is quoted:

"The co-precipitation of radium and barium presents some highly interesting features. It seems anomalous that radium should be precipitated together with barium at a concentration far below that corresponding to the solubility of pure radium sulphate (about $\frac{1}{100}$ that of BaSO_4). This action is exactly that to be expected if radium and barium were truly isotopic, but they have, of course, atomic numbers widely different, and can readily be separated by fractional crystallization of the halides. Moreover, since they are not isotopic, one would expect the theoretically unstable condition established by the co-precipitation to revert to equilibrium by the return of radium into solution. Such is not the case. On the other hand, the degree of co-precipitation is not so rigid as with true isotopes."

We are not inclined to regard the calcium precipitation as one involving specific adsorption, because of the large amount of calcium removed from solution. It would, indeed, be unusual for a precipitate to adsorb a molecular equivalent of another salt. Fairhall (14) has worked out a method for the quantitative determination of lead in urine, in which the calcium is precipitated as the phosphate in ammoniacal solution with the quantitative entrainment of lead. The mechanism of this reaction is readily explained on the basis of our findings. It would appear that the action of the parathyroid hormone, which is as specific for lead as for calcium, is also in harmony with the double lead-calcium salt conception.

SUMMARY.

Lead acetate added to serum with or without CO_2 tension precipitates a molecular equivalent of ultrafiltrable calcium and an amount of inorganic phosphorus required for the formation of trilead and tricalcium phosphate. Since the amount of calcium thrown out is independent of the pH (6.9 to 8.3) and of the concen-

tration of phosphate, carbonate, and calcium ions, the reaction is not immediately concerned with the solubility product of calcium carbonate or calcium phosphate. The reaction is a specific lead effect.'

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LACTIC ACID AND CARBOHYDRATE IN SEA URCHIN EGGS UNDER AEROBIC AND ANAEROBIC CONDITIONS.

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It has long since been established by Loeb (1) that unfertilized sea urchin eggs can exist for considerable periods of time when deprived of oxygen mechanically, or, when their oxidative processes are inhibited by the addition of cyanide to their medium. In the presence of potassium cyanide, as was shown by Loeb (1) as well as by the investigations of Warburg (2) and Meyerhof (3), the rate of aerobic respiration of the cells is greatly reduced or almost completely arrested. Such eggs will respire normally and will be capable of normal fertilization and subsequent development upon the addition of oxygen, or upon the removal of the cyanide by washing. This indicates clearly that the unfertilized eggs must possess an anaerobic metabolic process for the maintenance of life.

In an attempt to elucidate this phenomenon the analogy with the more recent findings or the anaerobic metabolism of tumor cells by Warburg (4), of yeast and other cells by Meyerhof (5) as well as of muscle cells deprived of sufficient oxygen, studied by Meyerhof (5) and by Hill and his school (6), inevitably presented itself. In all of the above investigations it was found that part of the energy required for the life processes was furnished by the anaerobic conversion of glucose to lactic acid. That lactic acid may be produced in sea urchin eggs was surmised by Loeb ((1), p. 31).

It was therefore decided to determine the lactic acid production in unfertilized sea urchin eggs under aerobic and anaerobic conditions.

EXPERIMENTAL.

Mature unfertilized eggs of *Arbacia punctulata* were obtained daily at the height of the season at Woods Hole (July and August). After removal of the ovaries from the females they were suspended in sea water, strained through cheese-cloth, and the eggs washed by suspension and sedimentation in three changes of sea water. The washed eggs were then suspended in a definite small volume of sea water, rapidly stirred, and 1.00 cc. samples removed for nitrogen analyses. The pipette used for this purpose was the same throughout the reported experiments. It was provided with a wide open tip to permit a rapid removal of the sample before sedimentation occurred. The nitrogen determination was carried out as follows: The 1.00 cc. sample was blown out into a 50 cc. Erlenmeyer flask and 1 cc. of concentrated sulfuric acid added followed by 1 to 2 cc. of water. On slight warming the eggs dissolved almost completely. The solution was transferred with washings to a 25 or 50 cc. volumetric flask and the contents made up to the mark with distilled water. An aliquot of the resulting, slightly turbid, solution was used for analysis by the micro Kjeldahl method of Koch and McMeekin (7). Direct Nesslerization could not be made on the digested material because of turbidity which invariably developed, and distillation or aeration was used to recover the ammonia. Subsequent Nesslerization yielded clear and easily readable solutions. For the sake of uniformity all of the analytic results are expressed in terms of mg. of lactic acid (or glucose) per 1 gm. of egg protein; the approximate factor of $\text{gm. of N}_2 \times 6.25 = \text{gm. of protein}$ was used. A similar method for the expression of analytic results on sea urchin eggs in terms of nitrogen was used by Meyerhof (3). This is believed to furnish a simpler basis for quantitative results than dry weight or the number of eggs.

Experiments with Potassium Cyanide.

The heavy suspension of the eggs in sea water described above was divided into two equal parts by volume. The same quantity of sea water was added to each, but the sea water added to one of these contained sufficient KCN to make its final concentration 0.02 per cent (approximately 0.003 M). The control and the

cyanide-treated egg suspensions were allowed to stand at room temperature (18–22°) for variable periods of time, 1 to 6 hours. If no large excess of sea water had been previously added, the suspension was triturated directly with the protein-precipitating reagent. In those cases where large amounts of sea water had been used, the eggs were sedimented by centrifugalization, and the supernatant fluid decanted and evaporated on a water bath to a conveniently small volume after the addition of a small amount of NaHCO_3 to render the solution distinctly alkaline. The sedimented eggs were triturated with the precipitating re-

TABLE I.
Effect of KCN on Lactic Acid Content of Unfertilized Eggs.

Experiment No.	Mg. lactic acid per 1 gm. egg protein.		Per cent increase.	Duration of experiment.
	Control eggs.	KCN eggs.		
				hrs.
1	3.27	5.60	72	4
2*	2.00	3.85	92	1
3*	2.05	4.20	105	6
4	2.80	4.71	68	2
5	2.95	7.14	142	2
6	4.39	5.14	17	2
7	5.13	8.64	69	6
8	2.44	6.13	151	1.5
Average	3.14	5.68	81	

* Experiments 2 and 3 were begun simultaneously with eggs taken from one mixed suspension.

agents while the concentrated supernatant fluid was treated with the same protein-precipitating reagents and the lactic acid was determined separately in the filtrates. A corresponding amount of KCN was added to the suspension of control eggs, immediately before the addition of the precipitating reagents, in order to insure the strict comparability of the two solutions in the subsequent chemical manipulations. The results shown in Tables I and II express the figures for the total suspensions. The removal of the protein was attended with certain difficulties. After considerable experimentation it was found that the Schenk method of precipitation with HCl and HgCl_2 , described by Cori (8), gave the

best results. The filtrates, freed of mercury by H_2S , were treated with $CuSO_4$ and lime for the removal of interfering substances, as recommended by Clausen (9), and lactic acid determinations were carried out by the Friedemann, Cotonio, and Shaffer modification (10) of Clausen's method. The results are shown in Table I. A relatively large increase in lactic acid production in unfertilized eggs under the influence of KCN was found in every experiment.

Experiments with Fertilized Eggs.

In these experiments the preliminary treatment of the eggs was the same as described above, except that special care was taken to select fully mature eggs which did not show any cytolysis. For this purpose the eggs from each ovary were examined separately and a sample was subjected to a preliminary fertilization test and only such eggs were taken as showed at least 60 per cent membrane formation after 10 minutes. The suspension of eggs was again divided into two equal parts, one of which served as a control, while to the other freshly obtained sperm was added. Both suspensions were allowed to stand in a water bath at $20-21^\circ$ in shallow dishes which were frequently and gently agitated to insure a plentiful supply of oxygen. Samples were examined under the microscope and the experiment terminated when about 90 per cent of the eggs had reached the two or four (or more) cell state of division. This period was usually 1 to 2 hours. Three experiments were terminated as soon as membrane formation had begun (after 7 to 12 minutes). In each case a corresponding amount of the same sperm was added to the control eggs immediately before precipitation of the protein.

It will be seen from Table II that while in each of the six fertilization experiments in which the embryo division was permitted to proceed to the two or four cell stage or beyond there was a definite increase in the lactic acid. This increase however is not nearly as great as in the case of the KCN-treated, unfertilized eggs. This can be easily explained by the enormously greater rate of oxidation proceeding in the dividing eggs as compared with that in the unfertilized eggs under the effect of KCN. The increase of the lactic acid in the eggs in which only membrane formation was allowed to take place was of the same order of magnitude as

in the divided eggs in two cases, while in one case an actual decrease was observed. The number of these latter experiments was too limited, however, to enable one to draw from them any definite conclusion.

Carbohydrate of *Arbacia* Eggs.

The question naturally arose as to the origin of the lactic acid. Meyerhof (3) in 1911 searching for the energy-yielding substance in the oxidative processes of the eggs of *Strongylocentrotus lividus*,

TABLE II.

Effect of Fertilization and Cell Division on Lactic Acid Content of Eggs.

Experiment No.	Mg. lactic acid per 1 gm. egg protein.		Per cent increase.	Duration.	Remarks.
	Control eggs.	Fertilized eggs.			
				min.	
1	2.80	3.50	25	88	96 per cent fertilization, 4-8 cells.
2	2.95	3.31	14	95	70 " " " 4-8 "
3	3.56	3.90	10	122	90 " " " 4-8 "
4	1.37	1.56	14	130	96 " " " 2-8 "
5	2.35	3.53	50	95	97 " " " 4 "
6	3.25	3.58	10	73	98 " " " 2-4 "
Average.	2.71	3.23	19		
7	3.05	3.73	+22	12	100 per cent membranes formed, no cell division.
8	3.00	3.25	+8	7	
9	3.82	3.40	-11	7	

could not demonstrate the presence of any carbohydrate (glycogen, or copper-reducing substances) in these eggs. Nor could he demonstrate any manifestations of the cleavage of protein during the oxidative processes. He did find, however, large amounts of fat, and he was forced to regard the fat as the substance oxidized.

It is of interest to note that Mathews (11) found in the eggs of the starfish (*Asterias forbesii*) large amounts of a phosphatide which yielded on prolonged hydrolysis with acid 10 per cent of a reducing sugar which he could not definitely identify as glucose. Mathews informed us that he had found carbohydrate-containing lipids also in *Arbacia* eggs.

Myers (12) reported the presence of 61 mg. of sugar per 100 cc. of "blood" in the Pacific sea urchin, *Strongylocentrotus francescames*.

The present writers made a number of exhaustive attempts in search of free reducing sugars in the eggs of *Arbacia*. Even when as many eggs as those obtained from 50 sea urchins were used, not a trace of copper-reducing material could be found in concentrated protein-free extracts. In the attempts to isolate glycogen from the eggs by the Pflüger method of extraction with concentrated alkali, small amounts of material were obtained, yielding an equivocal iodine test, and uncertain reduction tests after hydrolysis of this material with acid. Mr. K. Blanchard,¹ however, working later with dried eggs collected during the same summer at Woods Hole, found that if the eggs were previously

TABLE III.
Total Carbohydrate Content of Unfertilized Eggs.

Experiment No.	Mg. glucose per 1 gm. egg protein.	Duration of hydrolysis.
		hrs.
1	43	1
2	50	2
3	48	4
4	44	5
5	64	5

defatted, small amounts of glycogen could be obtained by Pflüger's method, which yielded on acid hydrolysis glucose, as shown by reduction tests and the melting point of the osazone.

In view of the uncertain results obtained by us in the search for glycogen it was decided to subject the eggs directly to acid hydrolysis in order to see whether combined carbohydrate was present in any form whatever. For this purpose the procedure described by Cori (13) was employed. Suspensions of eggs were hydrolyzed, with 0.5 to 1.0 N HCl in the water bath for 1 to 6 hours, the protein cleavage products removed with mercuric acetate, and the mercury removed with H₂S. In some instances colloidal iron was used instead of the mercury salt. The filtrates

¹ Personal communication.

so obtained showed in every case strong reduction with Fehling's and Benedict's reagents. When concentrated, good fermentation with yeast was produced and typical glucosazone crystals obtained.

In Table III some quantitative results are shown in terms of mg. of glucose per gm. of egg protein. The analyses were carried out with Folin's new sensitive sugar reagent (14) and with the use of Lloyd's reagent and permutit for the removal of interfering substances as recommended by Folin for urine.

It will be seen from Table III that the maximum amount of sugar is obtained already at the end of 1 hour's hydrolysis with acid.

DISCUSSION AND SUMMARY.

It has been shown in this investigation that mature unfertilized eggs of *Arbacia punctulata* produce lactic acid in the course of their normal aerobic metabolism. The concentration of lactic acid increases notably under the condition of anaerobic metabolism, in the presence of KCN. The concentration of lactic acid increases to a smaller extent after fertilization and in the early stages of cell division.

The reported results obviously furnish no information concerning the rate of formation of lactic acid under the aerobic and anaerobic conditions. It is possible that a greater concentration of lactic acid was found in the eggs treated with KCN, because under these conditions the oxidative processes, including the oxidation of lactic acid, are greatly retarded. Analogously the smaller increase in lactic acid in fertilized and actively dividing eggs may be due to the accelerated rate of oxidations in such eggs, causing the oxidative loss of some of the lactic acid.

The presence of free sugar could not be demonstrated in *Arbacia* eggs. The presence of glycogen was not definitely established by the present writers, but is reported to have been identified by another investigator in the same material. Hydrolysis of the unfertilized eggs with acid yielded readily a fermentable reducing sugar from which glucosazone was obtained. The amounts of total carbohydrate so found are more than ample to serve as the source of the observed lactic acid.

The anaerobic phase of metabolism of *Arbacia* eggs thus becomes quite analogous in its mechanism to that postulated for other tissues, such as tumors, yeasts, etc. ¹

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ON THE MECHANISM OF ENZYME ACTION.*

II. FURTHER EVIDENCE CONFIRMING THE OBSERVATIONS THAT ETHYLENE INCREASES THE PERMEABILITY OF CELLS AND ACTS AS A PROTECTOR.

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Lavoisier (1) first pointed out that during the decomposition of the sugar molecule in the course of alcoholic fermentation, the molecule not only splits in two parts to form alcohol and carbon dioxide, but he also clearly indicated that alcoholic fermentation involves the phenomena of reduction and oxidation.

Later workers have shown that the phenomenon of fermentation is not a simple process but one involving the presence of a number of intermediate products which may well differ in ease of fermentability. Indeed it seems probable that the generally accepted chemical formula for certain of these intermediate products does not express the true state of the reactive substances but that an intermediate "transportation form" (2) may well be the reactive material. One of us (Nord (3))¹ has presented elsewhere a critical review of the current theories. Doubts concerning the exact mechanism of enzyme action (4) and the question as to whether intermediate products are acted upon under "biological" or "unphysiological" conditions (5), as well as the question as to whether the so called activators of enzymes really increase enzyme activity or whether they simply protect and prevent

* Preliminary communication, Nord, F. F., *J. Biol. Chem.*, 1927, lxxiv, p. lviii; *Protoplasma*, 1928, iv, No. 4.

¹ This paper on "Chemical Processes in Fermentations" contains a general discussion of the theories of chemical processes involved in fermentation and a more complete discussion of the literature than it is possible to include in the present paper.

enzyme destruction, induced us to undertake the experiments reported in the present paper.

An investigation (6) of the physicochemical behavior of zymase solution, such as was prepared by Lebedew (7), led to the result that (a) it is possible to prepare zymase solutions which are capable of maintaining their biological activity. Data will be presented in this paper to show that these solutions maintained their full activity even after 65 days, although it has been generally assumed (8) that even at 0° the activity is lost within 48 hours. (b) It is not only possible to maintain the full activity of such a solution for practically an indefinite period, but it was shown that the enzyme solution, which behaves as a lyophile colloid, undergoes by means of appropriate peptization, a change of its surface forces, which is indicated by an increased surface tension and by decreased viscosity (6). Hence it was considered justifiable to conclude that the surface energy of the colloids concerned was increased, and this was reflected in the measurable increase of carbon dioxide production which was observed when the enzyme acted upon glucose.

After having reached, in certain cases, a production of 6.6 cc. of carbon dioxide per minute, the observation was made that this high fermentation activity was only maintained for a short time, the activity gradually falling to the average speed usually observed in enzymatic fermentation. This decrease could not have been due to an appreciable lack of fermentable sugar. It appears to be justifiable to interpret this observation as a further confirmation of an increased surface of the enzyme concerned. The increased surface is apparently much more sensitive toward the products of yeast metabolism. It might be assumed, therefore, that there are always enzymes present in a certain concentration, which are potentially capable of acting. However, since the reactivity of the enzyme is dependent upon its surface activity, it undergoes, immediately after the initial reaction, alterations which relatively decrease the velocity of the reaction, independently of the concentration of the reactant. In the course of the reaction, the proportion of damaged enzyme to active enzyme may be greatly increased. This conclusion indicates that there is a basis for the assumption that enzymes change their colloidal behavior even when bound to cells and under conditions where they may exert their activity.

Since we have, as yet, not been able to find a means whereby we can increase the surface of the acting enzymes within the living cell, we investigated the possibility of finding some method by which the original activity of the enzymes could be maintained, or by which the decrease of original activity could be delayed. Several considerations led to the idea that, independently of its reducibility, ethylene might be electrically charged on enzyme surfaces, in this way forming an adsorbed film, and since its opposed charges



are neutralized (or in any event altered) in the condition of adsorption, it not only would not hinder the formation of the enzyme-substrate complex, but might act as a protector of the surface upon which it was adsorbed. Our experiments seem to have justified this view-point, since we not only could protect the increased surfaces which, as mentioned above, were produced by the appropriate peptization of the enzyme solution, but also found it possible to show in a striking manner that a protecting film could be formed even on enzyme surfaces which were within the cell.

The application of ethylene disclosed another very remarkable quality of this gas. It was possible to show through the action of ethylene on living single cells or cells in tissues, as for instance, tobacco leaves, that it increases the permeability when the cell systems are exposed to its influence. Since no definite data in this respect were available in the literature, it was necessary to be sure of this effect on permeability, since, for example, there has never been an agreement as to whether ether, which is also a very effective narcotic, causes an increase or a decrease of permeability, even though it inhibits cell division (9).

Through the fact that the permeability was increased, it was further possible to show that a yeast suspension, after having been exposed for a longer time to a slow current of ethylene gas, shows in the beginning a strongly increased carbon dioxide production, followed in the main course of the sugar fermentation by a decreased production as compared to the controls. If, on the

other hand, an overcharging of the surface of the enzyme was avoided, the expected magnitude of the initial increased permeability was smaller, or even not noticeable, thus enabling the film to act as a protector, even during the fermentation of the first unit of glucose.

This observation led, on the one hand, to the connecting link between the alleged mechanism of enzymatic activity, and on the other hand, to the assumption, supported by this observation, that the physiological manner of acting of so called intermediate products in the biological form is fundamentally different from that in the unphysiological condition. It was Pfeiffer (10) who felt prompted to reemphasize recently that the association between enzymes and the substrate belongs in the group of true molecular combinations. If this be true, especially so far as zymases and fermentable sugars are concerned, it would be impossible to interpret the observations noted above, as well as those which are to follow.

Accordingly it was thought necessary to go a step further. In view of the well known fact that pyruvic acid exerts, probably because of its high degree of dissociation, an injurious effect on yeast cells, suspensions of yeast cells were allowed to act upon pyruvic acid solutions of different concentrations. It was not only possible to show that a 10 per cent suspension of yeast in a 1 per cent solution of pyruvic acid was capable of evolving practically the theoretical quantity of carbon dioxide within 26 hours, but the subsequent fermentation of 1 gm. of glucose in the original mashes showed, as expected, much less damaged enzyme in the case of the presence of a protecting film of ethylene than in its absence. It would be hard to understand how, in spite of the presence of a protecting adsorption film, a true molecular combination between enzyme and substrate could be assumed in our experiments, and it is much more plausible that these reactions also belong, in accordance with Gortner (11), to that group of reactions which may be regarded as governed by purely physical forces.

Mention has already been made of the effect of ethylene on single cells, and we believe that we have demonstrated that ethylene is not only capable of being charged as a protector or sensitive biological surfaces, but that due to adsorption it is able

to increase the permeability of cells. These observations were accordingly extended to cells in tissues, where, if the above observations were correct, the effect should be demonstrated perhaps even more strikingly. For this purpose we have investigated the effect on tobacco leaves, which, as we know from the investigations of Loew (12), contain a comparatively large quantity of catalase. The selected tobacco leaves, which were exposed to ethylene and then acted upon by a 6 per cent solution of hydrogen peroxide, evolved in fact a quantity of oxygen 15 to 20 per cent greater than did the leaves which were not exposed to ethylene.

These observations indicate furthermore that there occurs in the well known effect of the artificial ripening of fruits and vegetables (13) nothing less than an increase of the permeability of the cells, thus promoting the formation of the reactant-enzyme complex, and in this way advancing the hydrolysis of starch, from which are derived the sugar and other transformation products, and simultaneously, through the building up of the adsorbed protector film, enabling the enzymes to act for a prolonged time under conditions closer to ideal cases. In accordance with our considerations, there is no escaping the conclusion that the idiom "activation" is in this connection absolutely meaningless in its present use (14), especially since the phase of zymogens—if their existence according to our present uncertain knowledge may be considered as justified—might be regarded in our experiments as undoubtedly already having been passed.

EXPERIMENTAL.

Apparatus and Material.

The apparatus used for the fermentation experiments is the same as that described in a previous paper (6). The apparatus used in the catalase experiments consisted of a 150 cc., large mouthed, extraction flask, fitted with a 2-hole rubber stopper, a dropping funnel entering through one hole, and through the other a tube leading to a 3-way stop-cock, of which the two branches entered two burettes inverted in a dish of water. By applying suction to the burette tips, they can be easily refilled, and the

use of the 3-way stop-cock permits the taking of consecutive readings without interference. The flask in which the hydrogen peroxide was acted upon by the catalase was kept in a constant temperature bath.

The ethylene used was a commercial product compressed in tanks, prepared for anesthetic purposes, and had a purity of 99.15 per cent.

The dextrose used was a chemically pure product. The yeasts were: (a) pure top yeast furnished by the Fleischmann Company, especially purified, and furnished at regular intervals, which insured its fresh condition for use in experiments; (b) bottom yeast supplied by the Minneapolis Brewing Company at regular intervals (after having been carefully washed and compressed).

Yeast juice was prepared from (b) in the following manner: The washed and carefully pressed yeast was dried under a vacuum of 34 mm. at 30° for 24 hours, in layers not exceeding 4 to 5 mm. in thickness. The dried yeast was then milled to grains of the average size of 1 to 2 mm. A 100 gm. portion of this yeast was introduced into 300 cc. of tap water, which had been previously saturated at 30° with carbon dioxide. The suspension was macerated in a mortar with a pestle until it was of a fairly uniform consistency, and was then submitted to maceration for 6 hours at 25-26°. In order to obtain a clear solution of the zymase which had partially come out of the cells, the juice was centrifuged for half an hour at approximately 4000 R.P.M. The solution was then carefully removed with a pipette from the sediment and submitted to a second centrifuging for the same length of time. The final juice had a tea color and was water-clear. Examination under the microscope indicated that it was absolutely free of cell particles, and it showed no spontaneous fermentation.

Zymin was also prepared from (b). 1000 gm. of yeast were suspended in 1000 cc. of water and most of the proteins of the plasma membrane were precipitated in an irreversible manner by adding, within 20 minutes, 6000 cc. of acetone. The product was allowed to stand 15 minutes in the acetone, then filtered, washed carefully with ether, and dried at 35° for 24 hours.

The pyruvic acid used was purified by distilling it *in vacuo* (at 8 mm. and 55°) in an atmosphere of carbon dioxide.

The hydrogen peroxide used was Merck's superoxol, containing about 30 per cent H_2O_2 .

The tobacco leaves used were of the Blue Pryor strain.²

Method.

The solution or suspension used was always made up with a carbon dioxide-saturated tap water of an average initial pH of 6.0, containing 50 parts per million of calcium. The saturation and other preparatory operations were always carried out at the thermostat temperatures in order to avoid any influences due to contracting or expanding volumes.

The parallel experiment was conducted by bubbling ethylene through an opening of a 2 mm. tube, at a speed of 70 bubbles per minute, at a pressure of a 100 mm. water column for the time noted in each experiment.

The fermentations were carried out by the addition of 1 gm. (or amounts as stated in the experiments) of glucose. This gm. would furnish, if completely fermented, 282.4 cc. of carbon dioxide at 760 mm. and 30.5°. If pyruvic acid were completely fermented, 0.1 gm. would give 28.25 cc. of carbon dioxide under the same conditions. In most cases 20 cc. of the juice or of the yeast suspension, of which the type and concentration are noted in the discussion of the experiments, were used.

In all the fermentation experiments there was introduced quantitatively a second, and in some cases, a third gm. of glucose. These additions were made after the rates of the two fermenting mashings were approaching an identical magnitude.

During the main course of the fermentation, all readings were made at 1 minute intervals. In drawing the curves, however, for technical reasons, only greater intervals were plotted.

DISCUSSION.

1. Experiments on Yeast Juice.

Ethylene was bubbled through 20 cc. of freshly prepared juice for 30 minutes, and this solution was preserved overnight at

² The authors are indebted to Dr. James Johnson of the University of Wisconsin, for courtesies extended to them in supplying fresh tobacco leaves as required, and to Mr. R. B. Zimmermann, superintendent of the Minneapolis Brewing Company, for bottom yeast furnished.

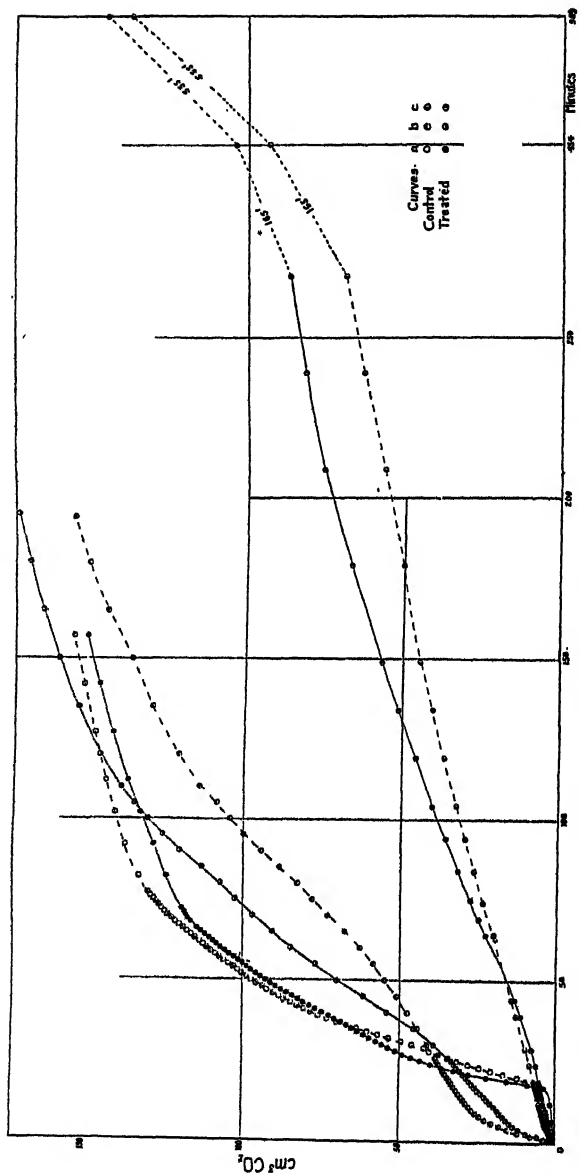


FIG. 1. (Experiment 1.) Fermentation of glucose with 20 cc. of yeast juice and with 20 cc. of yeast juice treated with ethylene. Curves (a) initial 1 gm. of glucose, curves (b) a second 1 gm. of glucose, curves (c) a third 1 gm. of glucose.

—10°. The control was prepared in a similar manner except for the gas treatment.

1 gm. of glucose was added to each solution just before commencing the measurements. The course of the fermentation is shown in curves (a), Fig. 1. After a period of 157 minutes a second gm. of glucose was added, and the course of the reaction, illustrated in curves (b) and (c), Fig. 1, shows the course of fermentation after the third gm. of sugar had been added. The difference in the rate of carbon dioxide produced is practically all in favor of the enzyme, treated with ethylene, especially during the course of the fermentation of the second and third gm. of glucose.

The juice used in the preceding experiment had been divided into two parts, and the results shown in Fig. 2 are those obtained 65 days after the preparation had been made.

Three different factors enter into this experiment. First, the once frozen juice was kept permanently at —10° in order to avoid possible influences upon the physicochemical conditions of the enzymes due to repeated thawing. Second, in view of the well known antiseptic properties of toluene (15), 1 per cent of this was added to the mash ready for fermentation. Third, ethylene was bubbled through the treated enzyme solution for only 10 minutes.

It will be noted in comparing Figs. 1 and 2, that the rate of the fermentation shows not only no decrease when viewed from the standpoint that only 15 cc. of juice and 0.75 gm. of glucose were used in this experiment, but even indicates that the absolute rate of carbon dioxide production would have been increased if the data of observations had been calculated on the magnitude of the preceding experiments (20 cc. of juice + 1 gm. of glucose). Since it is generally assumed that the loss of the activity of a zymase solution is mainly due to the action of proteases on zymase, and that the temperature and time factors are very uncertain, the results of this experiment show conclusively that it was possible to reduce the protease action so that its influence was not detectable. In spite of the fact that our solution was absolutely free of cells, there was no assurance that it was absolutely free of proteases. The remarkable maintenance of the original activity appears therefore to be due to a very significant equalization of the action of proteases by the increased surface activity of lyophilic

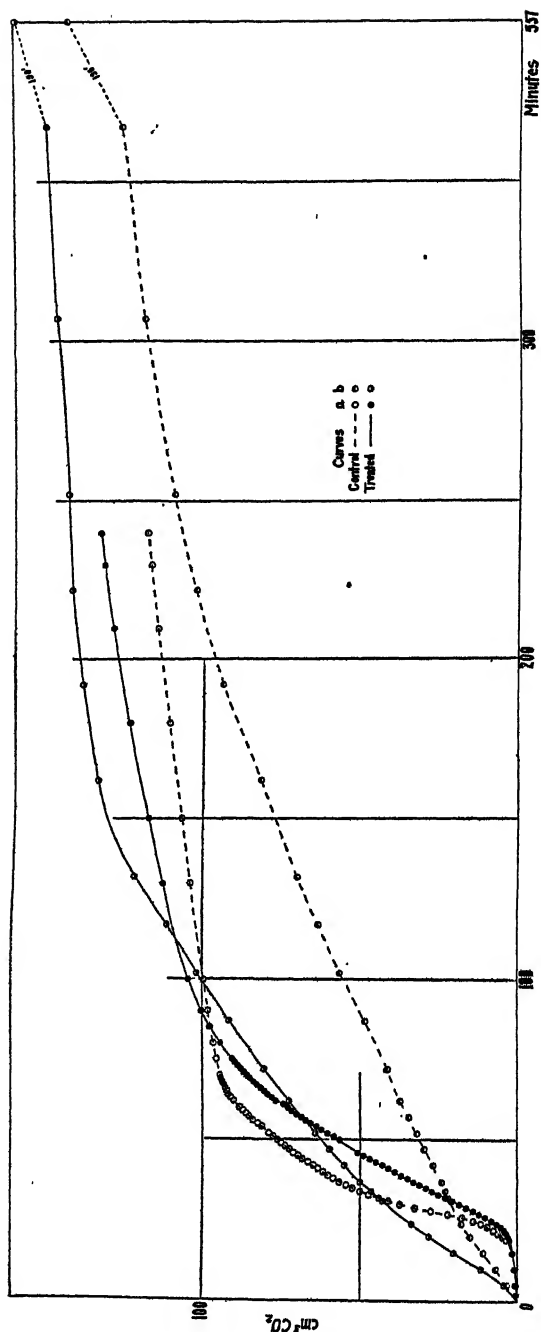


Fig. 2. The fermentation of glucose by 15 cc. of yeast juice preparations identical with those used in Experiment 1 (Fig. 1) after the juice had stood for 65 days at -10° . Curves (a) initial 0.75 gm. of glucose, curves (b) a second 0.75 gm. of glucose.

colloidal zymases. Therefore the conclusion might be drawn that in the complete absence of proteases, zymase solutions could be obtained which show not only practically indefinite keeping qualities, but are capable, in the beginning and within certain ranges, of showing a pronounced increase of their capacity to act on fermentable substrates.

The inspection of the curves indicates clearly the influence of a film adsorbed on the surface of the enzymes. This makes improbable two hypotheses; first, that in the case of fermentation, the enzyme-substrate complex may be considered as a true chemical combination, and second, that a film formed on the surface of a colloidal enzyme either by a gas, liquid, or solid compound, because of its reducibility, "activates" the processes. The main factor governing the effect is rather a physical one and depends on the capacity of the substances to be adsorbed, and in this way to interact between enzyme, substrate, or transformation products.

2. Experiments with Living Yeasts.

The preceding experiments led to the idea of applying our experiences in protecting enzyme surfaces to those in living yeast cells.

(a) *Bottom Yeast.*—The bottom yeast which served as a source for the zymase solutions in the above experiments was now used.

The purpose of these experiments was not only to show the possibility of the formation of a film on the surface of enzymes, bound on living cells, but to prove that it is a film, the formation of which is relatively independent of the period during which ethylene is bubbled through the preparation.

Three parallel experiments are shown in Figs. 3, 4, and 5, in each of which 20 cc. of a 10 per cent suspension acting upon each gm. of glucose were used. In the first experiment (Fig. 3) the enzyme surface was charged by bubbling ethylene for 30 minutes and kept at $+5^{\circ}$ to $+8^{\circ}$ for 12 hours. For the second (Fig. 4) the charging with ethylene was terminated after 10 minutes and the fermentation started subsequently. For the last experiment (Fig. 5) the ethylene gas was bubbled through the suspension for only 1 minute, followed by the immediate addition of sugar.

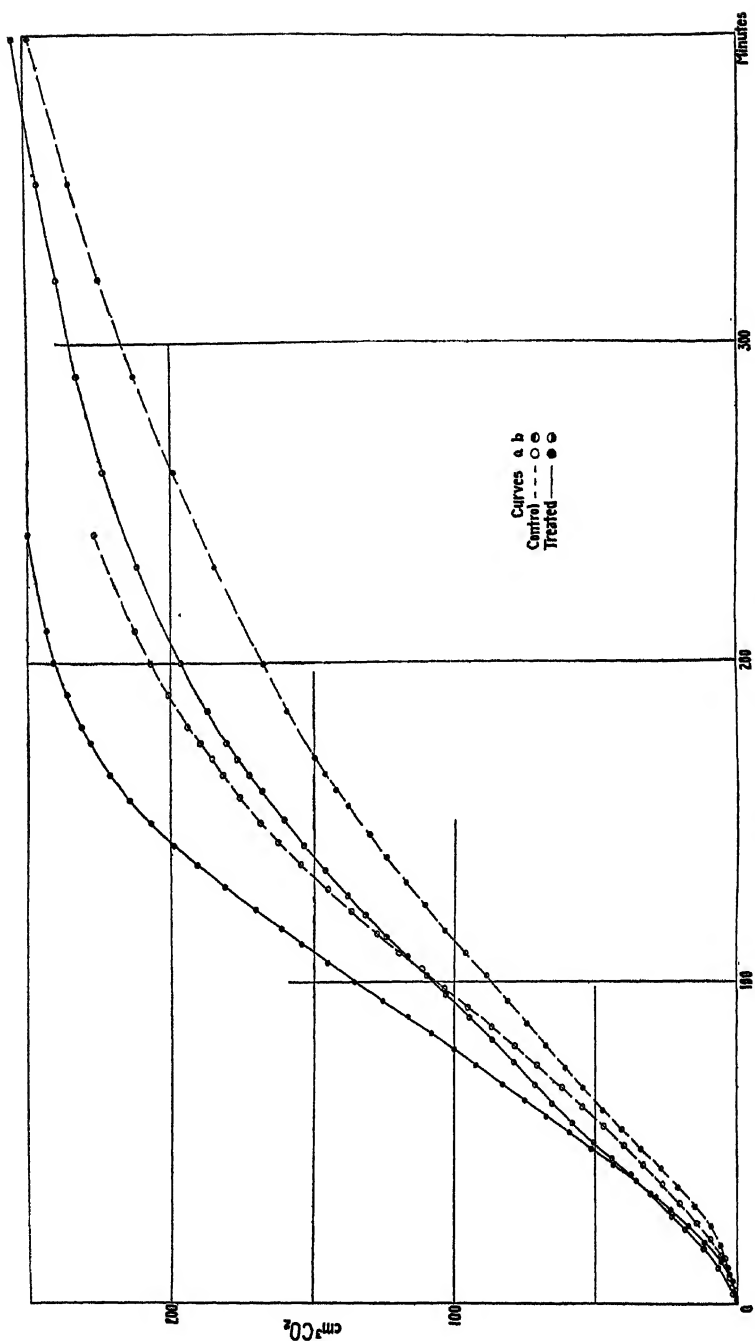


FIG. 3. The fermentation of glucose by a 10 per cent bottom yeast suspension and with a similar suspension treated for 30 minutes with ethylene. Curves (a) initial 1 gm. of glucose, curves (b) a second 1 gm. of glucose.

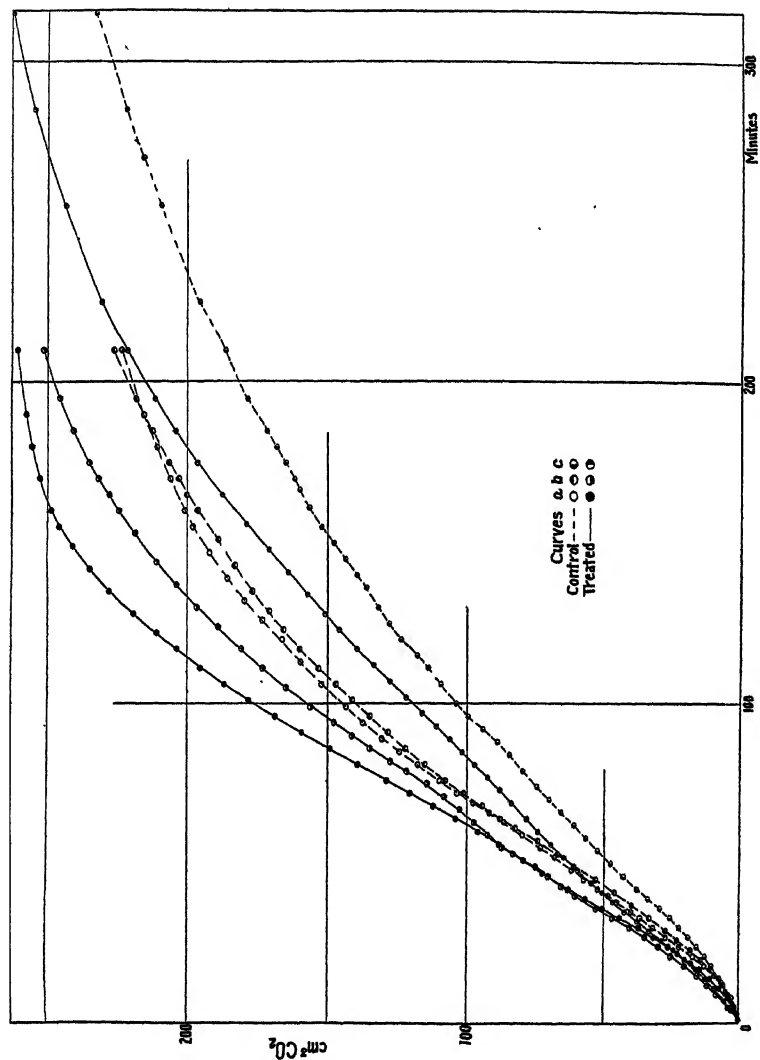


Fig. 4. The fermentation of glucose with a 10 per cent bottom yeast suspension and with a similar suspension treated for 10 minutes with ethylene. Curves (a) initial 1 gm. of glucose, curves (b) a second 1 gm. of glucose, curves (c) a third 1 gm. of glucose.

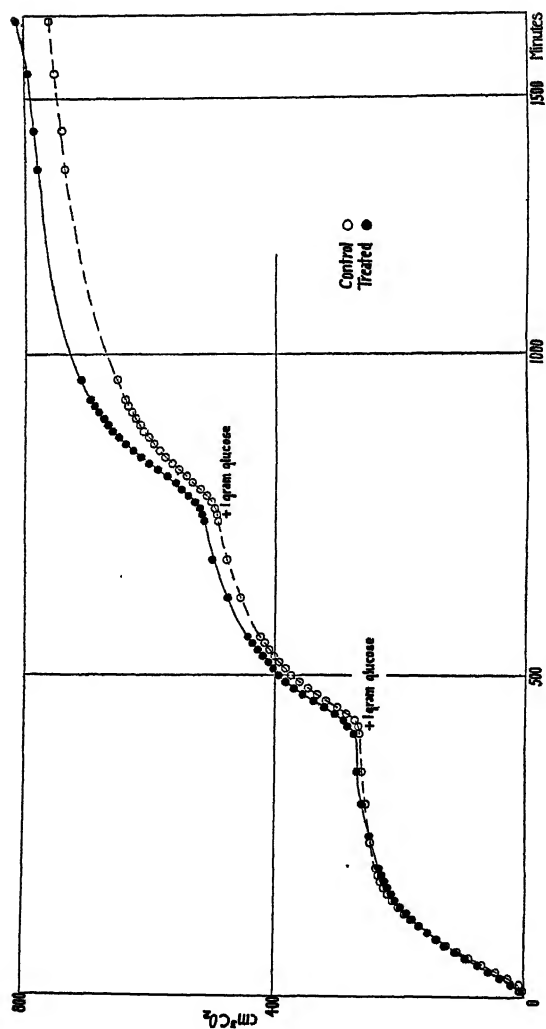


FIG. 5. The fermentation of glucose with a 10 per cent bottom yeast suspension and with a similar suspension treated for 1 minute with ethylene.

In all three experiments it is clearly demonstrated that the permeability of the treated yeast used was greatly increased. The decrease of the activity of the yeast enzymes, which is mainly due to the increasing concentration of the alcohol, was delayed not only during the fermentation of the first gm. of sugar, but more so during the course of the fermentation of the second and third gm. additions. In the case of the yeast treated with ethylene, this decrease is very much less than in the controls.

Comparison of the curves in Fig. 3 with those in Figs. 4 and 5 shows a lower fermentation rate, especially in the beginning, probably due to the fact that the enzyme surface was overcharged and the coating, according to Ramsden (16), passes gradually back into solution.

The fermentations represented by Fig. 5 picture the proceedings by a cumulative curve which indicates clearly the effect of the protecting surface against the transformation products, proving simultaneously that the ideal ratio of 1 between active and inactive enzymes falls much faster in those experiments where the enzyme is not protected.

(b) *Top Yeast*.—Similar experiments were carried out with a top yeast, whereby the general experimental conditions remained unchanged, except that the suspension used (Fig. 6) was in contact with ethylene 1 minute, and in the other case (Fig. 7) for 10 minutes.

However, in the case of fermentations represented by Fig. 7, a significant change was made in the procedure preceding the subsequent glucose additions. In order to make sure that *chemical* properties of the ethylene that may be present and dissolved in the mash were not responsible for the increasing delay of zymase inactivation, the liquid of the fermentation mash was removed by washing the yeast in the centrifuge after each finished fermentation, the washing being repeated three times with 70 to 80 cc. of water and the fermentation then being resumed on the addition of glucose to this washed suspension of original yeast.

The course of the reaction represented in both Figs. 6 and 7 confirms, without restrictions, the conclusions drawn from the fermentations carried out with bottom yeast.

(c) *Dough Fermentations with Top Yeast*.—Inasmuch as an increase of permeability in enzyme-containing cells is supposed

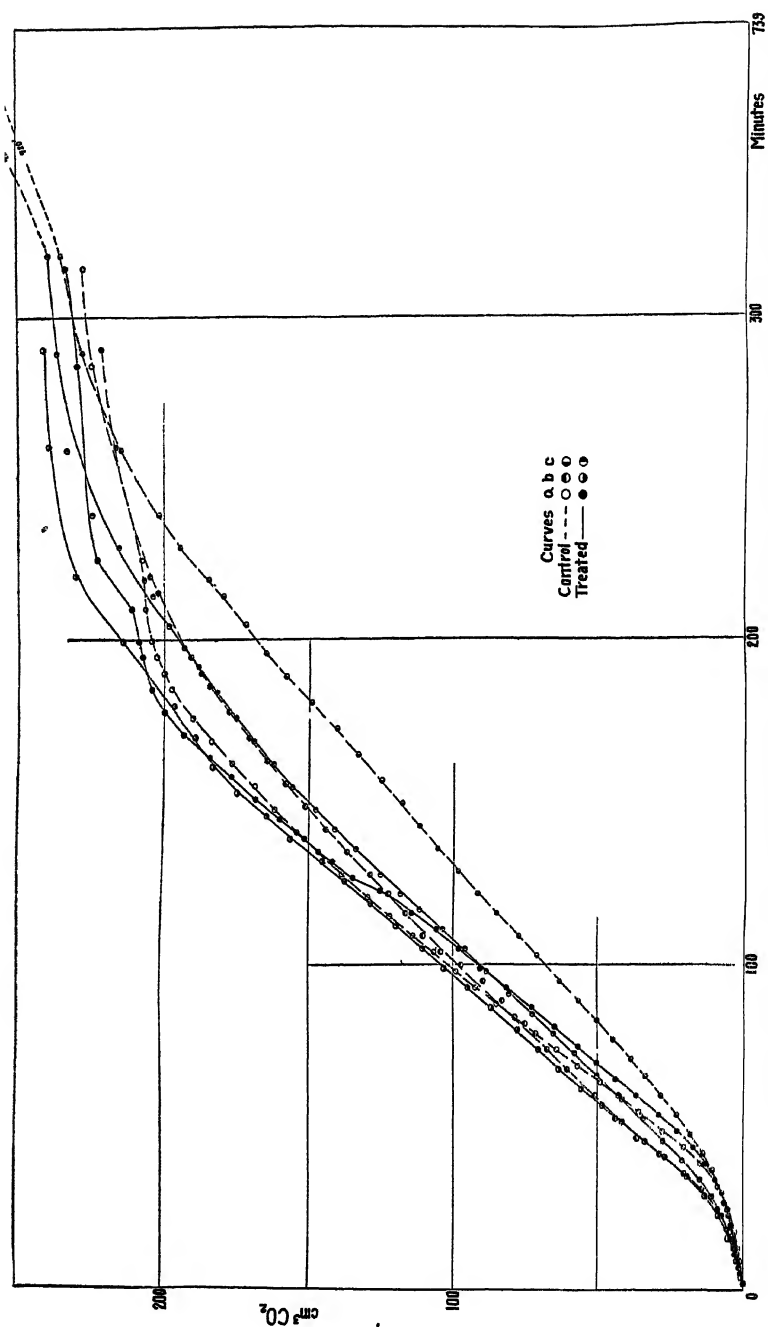


FIG. 6. The fermentation of glucose with a 10 per cent top yeast suspension treated for 1 minute with ethylene. Curves (a), (b), and (c) as in Fig. 4.

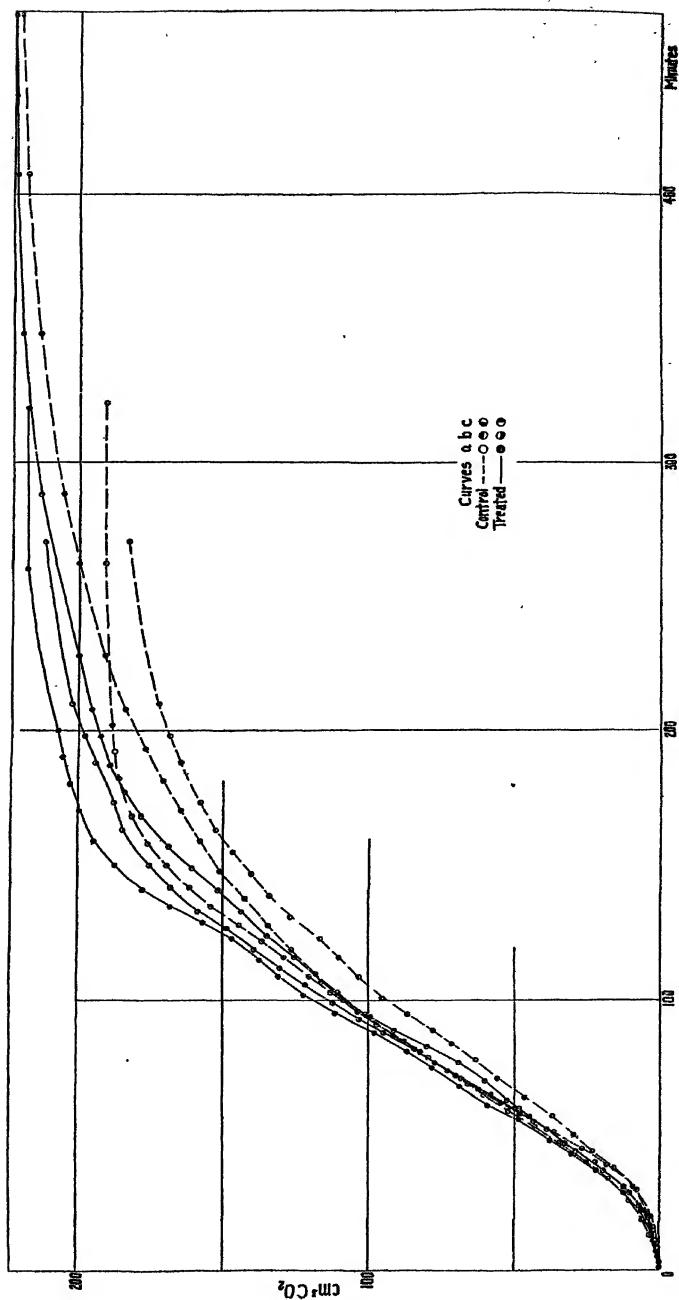


Fig. 7. The fermentation of glucose with a 10 per cent yeast suspension and with a similar suspension treated for 10 minutes with ethylene. Curves (a) initial 1 gm. of glucose, curves (b) the washed yeast cells from the experiment represented by curves (a) and a second 1 gm. of glucose, curves (c) the washed yeast cells from the experiment represented by curves (b) and a third 1 gm. of glucose.

to favor the formation of all enzyme-reactant complexes, it was thought advisable to carry out experiments in which a combination of enzymatic reactions takes place. A dough fermentation was considered the most suitable, since here a starch-gluten mass is acted upon by diastase on the one hand, and the transformation products of the diastatic activity as well as added sugar are acted upon by the invertase and zymases present on the other hand. In our experiments it was not possible to demonstrate that there was an augmentation of the diastase-starch reaction in the presence of ethylene. Such a possible influence of the ethylene could only take place in so far as free ethylene remained dissolved in the water in which the yeast was suspended.

A series of baking experiments³ was carried out. Each batch of dough was made according to the following formula: 350 gm. of wheat flour, 10.5 gm. of sucrose, 5.25 gm. of salt, 10.5 gm. (or less) of yeast suspended in 50 cc. of water, 162.5 cc. of water, 9 gm. of compounded lard. This was fermented for 3 hours and 50 minutes. The procedure used is that which is considered as standard in baking tests.

The baking experiments carried out with 60 loaves demonstrated that there was a remarkable increase in the volume of those loaves which were prepared with the same quantity of yeast in which the cell permeability was increased by ethylene treatment. In good agreement with this fact, it was noted that the same loaf volume as the control was obtained by using 10 to 15 per cent less yeast, when the yeast had been previously treated with ethylene so that its permeability had been increased, or its enzymes were protected.

3. Experiments with Tobacco Leaf Catalase.

It was considered desirable to extend the investigations regarding the possible increase of permeability to cells in tissues. Tobacco leaves were accordingly chosen, since they contain, as already noted, a comparatively large quantity of catalase.

The experiments were carried out by shredding representative leaves of the Blue Pryor strain, and placing 5 gm. samples with 60 cc. of water in the reaction flask. The pulp, in curves (a)

³ These tests were carried out by Mr. C. C. Fifield.

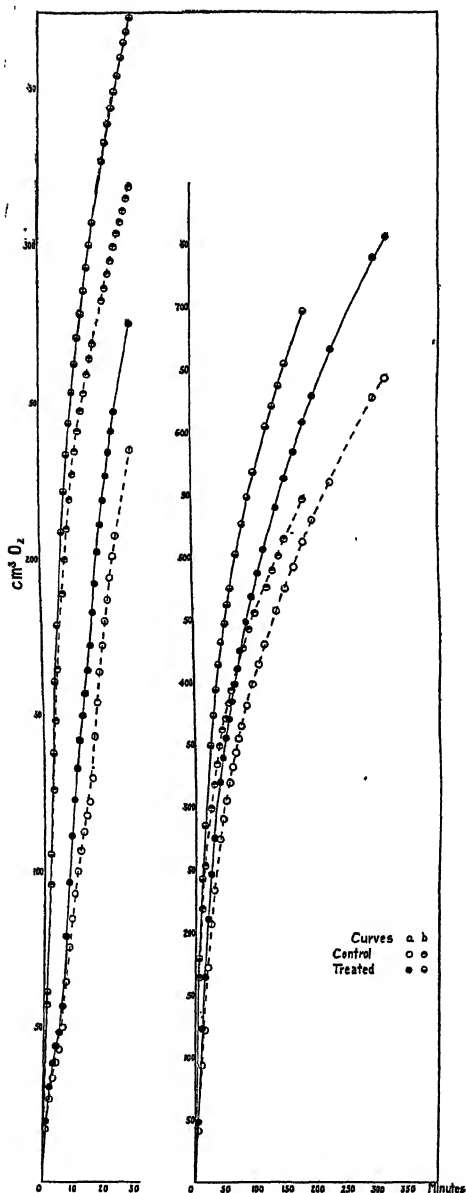


FIG. 8. The decomposition of H_2O_2 by catalase of tobacco leaf pulp and by tobacco leaf pulp treated with ethylene. The curves on the left show the cc. of O_2 evolved during the first 30 minutes of the reaction, those on the right the entire course of the reaction.

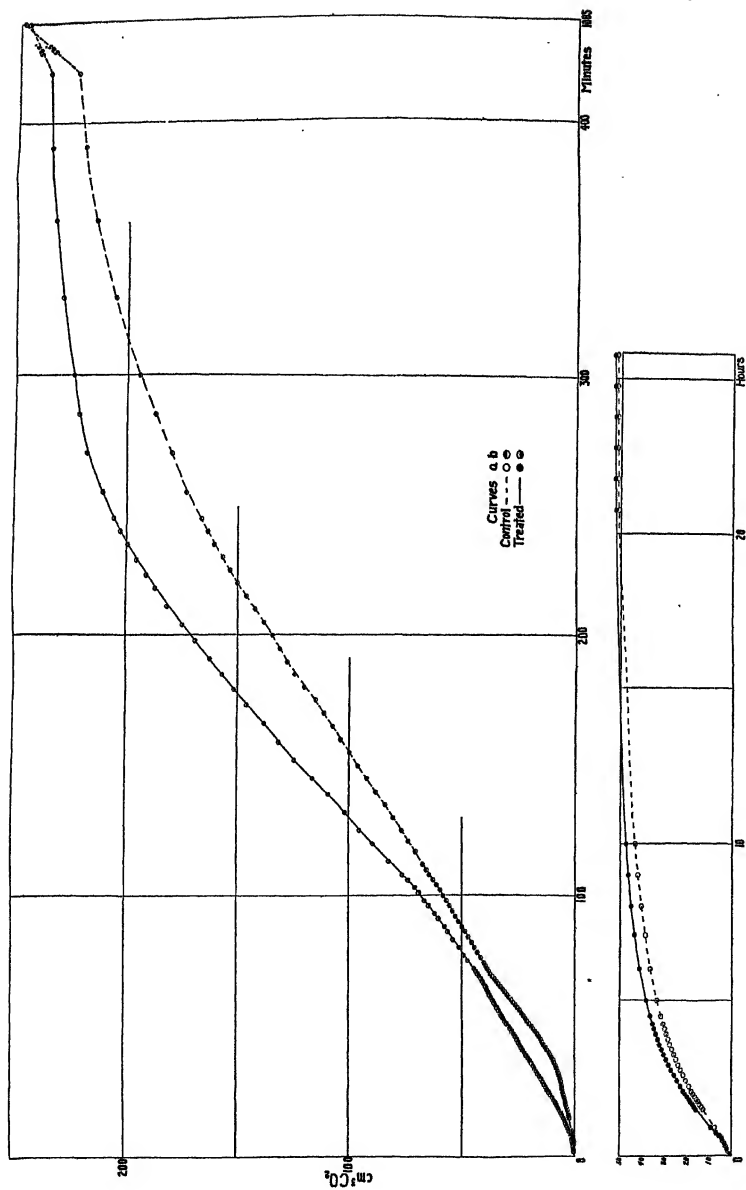


Fig. 9. The bottom curves (a) represent the fermentation of a 1 per cent pyruvic acid solution with a 10 per cent suspension of top yeast and with a similar suspension of top yeast treated with ethylene. The top curves (b) represent the fermentation of 1 gm. of glucose with the washed residual yeasts centrifuged after the completion of the above experiment.

and (b), Fig. 8, was treated by bubbling ethylene gas through it for a few minutes in the usual manner.

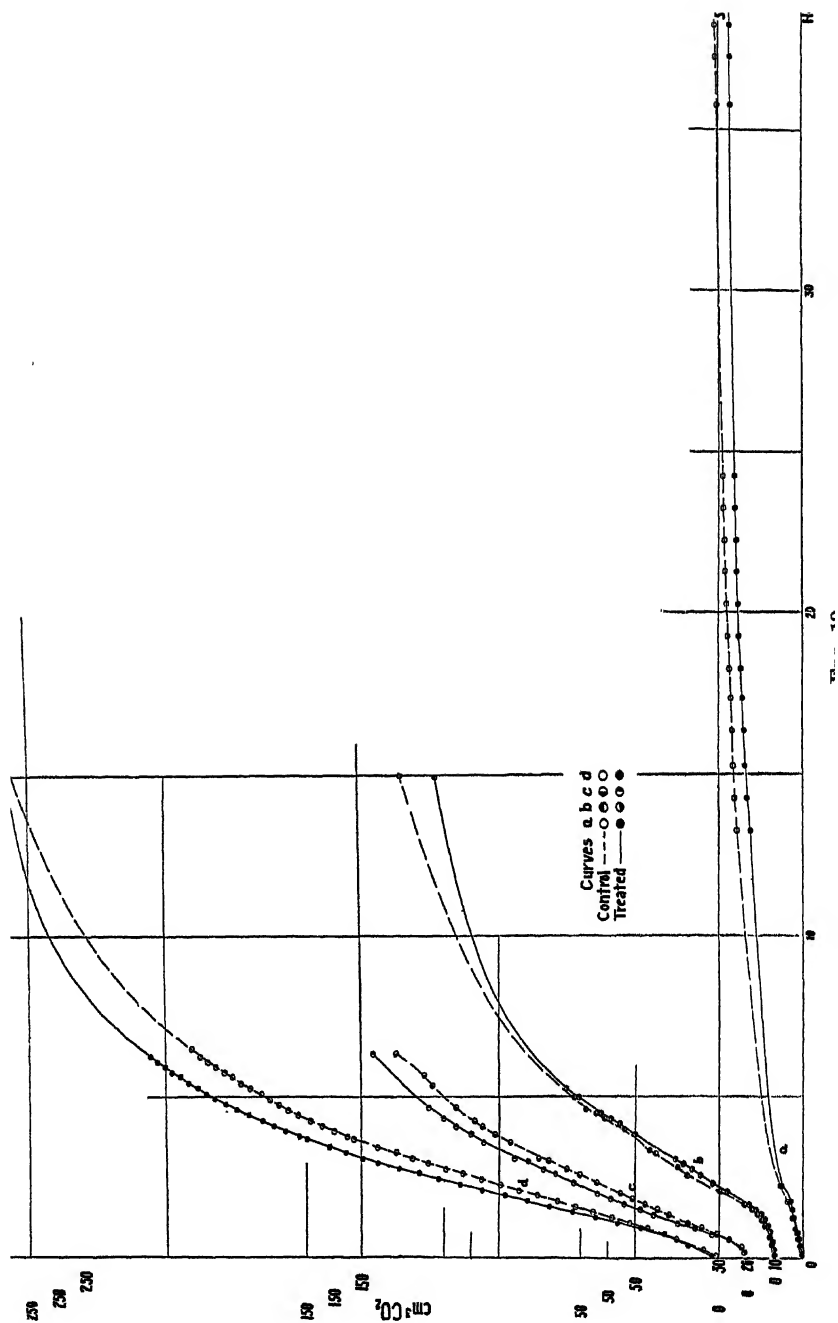
It is easy to see from the curves that the treated leaves have a higher production of oxygen than the controls, and also that there is an increased oxygen production due to the increased permeability from the start. This may be seen by noting the curves on the left, which picture the course of the reaction during the period of the first 30 minutes. It can be easily recognized that an increased permeability occurred from the start, as the differences in the oxygen evolution during the entire course of the reaction are overwhelmingly in favor of the treated pulp.

4. Experiments with Living Yeast on Pyruvic Acid.

The experiments with pyruvic acid were carried out from two view-points. First, in spite of the fact that the earlier contradictory opinions (17) regarding the fermentability of pyruvic acid have been cleared up, there is, judging from a search of the recent literature (18), no agreement concerning the rate of fermentation of this important compound. Second, it was regarded as necessary to demonstrate that the yeast can be protected also against the aforementioned injurious effect of the unphysiological acid; *i.e.*, that the capacity of pyruvic acid to inactivate yeast can be decreased even during relatively long contact of the yeast with the acid.

It is believed that these view-points are met by presenting the following experiments.

In the experiment shown in Fig. 9, 10 cc. of a 20 per cent top yeast suspension were treated by bubbling ethylene through it for 20 minutes. 10 cc. of a 2 per cent pyruvic acid solution were then added, reducing in this way the yeast concentration to 10 per cent and that of the acid to 1 per cent. The bottom curves (a) show the fermentation of pyruvic acid, indicating that not only the permeability of the treated yeast was remarkably increased, but that in 26 hours about 95 per cent of the pyruvic acid had been fermented. The yeast was then washed three times with 70 to 80 cc. of tap water, separated by centrifuging, then suspended in carbon dioxide-saturated tap water, and used in the fermentation of 1 gm. of glucose (top curves (b)). The



significance of the protection obtained by the use of ethylene is evident.

In the experiment represented by Fig. 10, the conditions were somewhat altered in that 0.5 per cent pyruvic acid was fermented in a 5 per cent yeast suspension (curves (a)). The period of ethylene treatment was 10 minutes.

The pyruvic acid fermentation was followed, without washing the yeast, by three subsequent sugar fermentations, the course of which may be easily recognized by the steeper curves (b) and (c) (representing 0.5 gm. of glucose) and curves (d) (representing 1 gm. of glucose).

In order to ascertain the effect of a more unfavorable relation between yeast available for fermentation and acid to be fermented, 1 per cent pyruvic acid was fermented by a suspension containing 2.5 per cent yeast, which was previously treated with ethylene for 20 minutes. After the completion of the pyruvic acid fermentation (curves (a), Fig. 11), the yeast was carefully washed three times, each time with 70 to 80 cc. of tap water, again suspended in carbon dioxide-saturated water, and allowed to act upon 1 gm. of glucose (curves (b)). Compared with Fig. 9, the inspection of the curves conveys evidence to the effect of a smaller number of active cells during the course of the sugar fermentation, which is, moreover, more vigorous in the experiment carried out with protected enzymes.

The ability to resist the harmful effects of pyruvic acid and the possible high content of cozymase shown by the top yeast used are also evident by the inspection of the curves (a) and (b) of Fig. 12, where experiments with bottom yeast are recorded.

The experiments shown in Fig. 12 were carried out by using suspensions containing 10 per cent bottom yeast acting on 1 per cent pyruvic acid. The upper curves (a) indicate the course of

FIG. 10. Curves (a) represent the fermentation of a 0.5 per cent pyruvic acid solution with a 5 per cent suspension of top yeast and with a similar suspension of yeast treated with ethylene, curves (b) a subsequent fermentation of 0.5 gm. of glucose added following the completion of the pyruvic acid fermentation, curves (c) a second 0.5 gm. of glucose following the experiment represented by curves (b), and curves (d) a final fermentation of 1 gm. of glucose added following the experiment represented by curves (c).

the pyruvic acid fermentation and show that it yields not more than one-fourth to one-fifth of the theoretical amount of carbon

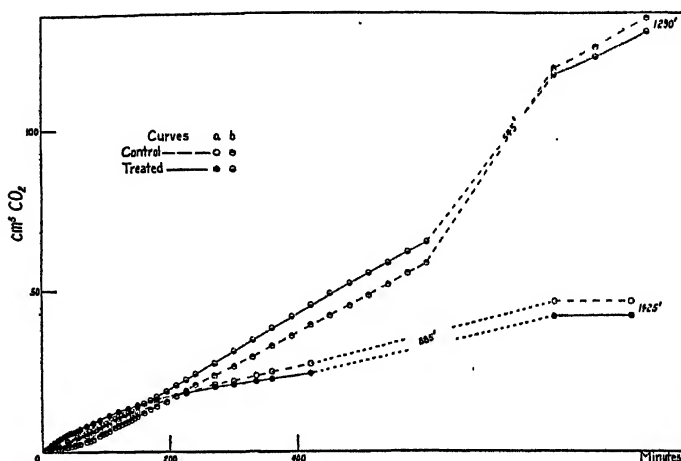


FIG. 11. Curves (a) represent the fermentation of a 1 per cent pyruvic acid solution with a 2.5 per cent suspension of top yeast and with a similar suspension of yeast which had been treated with ethylene, curves (b) the fermentation of 1 gm. of glucose with the washed yeast residues from the experiment represented by curves (a).

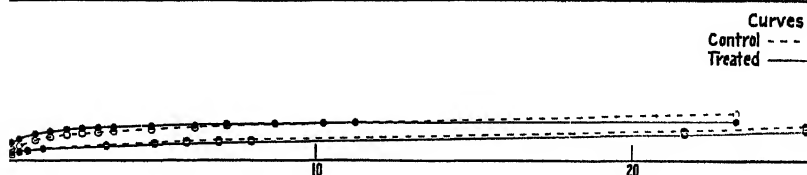


FIG. 12. Curves (a) represent the fermentation of a 1 per cent solution of pyruvic acid with a 10 per cent suspension of bottom yeast and with a similar suspension of bottom yeast treated with ethylene, curves (b) the fermentation of 1 gm. of glucose with the washed yeast residues from the experiment represented by curves (a).

dioxide. After a careful washing of the used yeast, its fermentability was tried out on glucose, but curves (b) indicate that the yeast was practically killed.

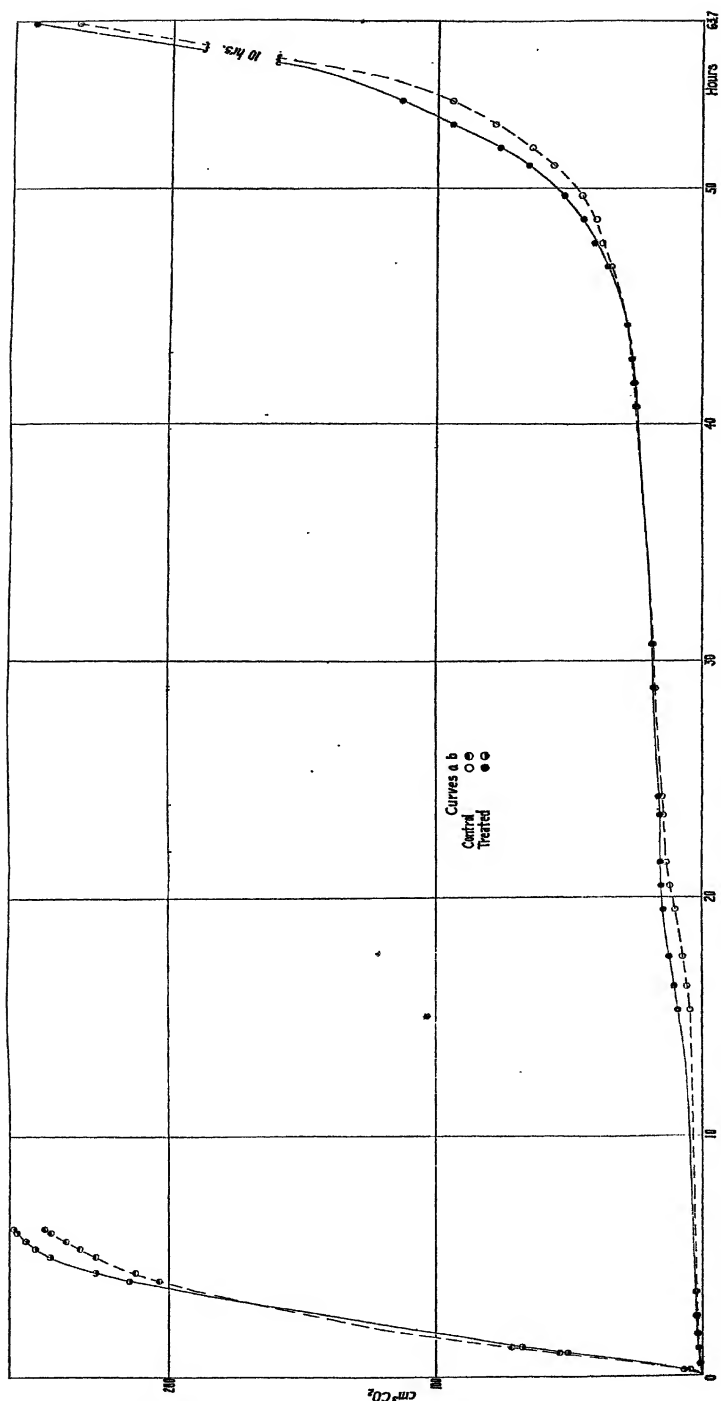


Fig. 13. The fermentation of glucose with a zymoin preparation and with a zymoin preparation treated with ethylene. Curves (a) the fermentation of an initial 1 gm. of glucose, and curves (b) the fermentation of a second 1 gm. of glucose by the same zymoin preparation.

5. *Experiments with Zymin.*

Fig. 13 represents the course of an experiment carried out with zymin, the preparation of which is indicated on p. 32. The fermenting mash consisted of 1 gm. of zymin in 20 cc. of tap water, to which was added 1 gm. of glucose, after exposure to ethylene for 10 minutes. The inspection of the curves discloses a good agreement with previous experiments; however, the extraordinarily long induction time in the first pair of curves (a) is completely overshadowed by a fermentation rate, in the course of the fermentation of the subsequent gm. of glucose, only slightly lower than that produced by a mash made up with the same, but living yeast.

In view of the fact that Harden (19) regards zymin as "quite incapable of growth or reproduction, but produces a very considerable amount of alcoholic fermentation," this observation might be regarded to be in indirect agreement with the conclusion of Giaja (20), who regards it as not yet proved that the fermentation activity of living yeast cells has to be ascribed exclusively to the zymases present.

6. *Fermentation of Calcium Hexose Diphosphate by Living Yeast.*

Up to date it has been considered as proved that zymophosphates are unfermentable by living yeasts (21), and even recently this observation was thought to be confirmed.

The surprising observations on our top yeast suggested that we should again take up this question, especially since the unfermentability of these products by living yeasts has given rise, during the last few years, to numerous speculations. The I. G. Farbenindustrie A. G. in Elberfeld kindly put at our disposal a purified, insoluble calcium salt, which according to a communication of Dr. Hoerlein, contained only 13.64 per cent calcium and 11.08 per cent phosphorus, instead of 18.41 per cent calcium and 14.28 per cent phosphorus. From this salt the easily soluble magnesium salt was prepared, which is mentioned for the first time by Young (22). After the fermentation for 44 hours of a 2 gm. suspension of the calcium salt in 20 cc. of a 20 per cent top yeast suspension, there was obtained a quantity of carbon dioxide which was practically equivalent to the amount of sugar which,

in accordance with the analysis, could be considered as uncombined.

The fermentation of the magnesium salt under the same conditions yielded, after 24 hours, practically no carbon dioxide.

CONCLUSIONS.

1. Zymase solutions were prepared which not only maintained their full activity for 65 days, but showed an increased activity.

2. Ethylene produces the effect of increasing the permeability of single cells and cells in tissues.

3. Enzymes outside of or bound to cells can be charged with an adsorbed film which may act as a protector against damaging transformation products.

This work has been aided as the result of a fellowship from the International Education Board to the senior author, who takes pleasure in acknowledging his indebtedness to Dr. R. A. Gortner and Dr. C. H. Bailey, of the Division of Agricultural Biochemistry, for the hospitality and consultations which he has enjoyed while at the University of Minnesota.

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IMPROVED PROCEDURE FOR THE EXTRACTION OF THE OVARIAN HORMONE.

II. SOME CORRECTIONS AND ADDITIONS.*

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During the 2 years following the publication of our improved procedure for the extraction of the ovarian hormone from liquor folliculi, many new and interesting features have been encountered. The purpose of the present paper is to make such corrections of the earlier description as are necessary and to add to the procedure the steps that we take to transfer the partially purified hormone to aqueous solution.

In order to attain greater clarity in recording the essential details of the modifications, the main points of the procedure of our earlier paper (Ralls, Jordan, and Doisy, 1926) are given. After distillation of the alcohol used for extraction of the liquor folliculi, the dry residue is dissolved in dilute alkali. This aqueous solution is then extracted with ethyl ether (Stage A), the ether distilled, and the residue transferred with 95 per cent alcohol to a separatory funnel. Enough water is added to reduce the concentration of alcohol to 70 per cent, and some of the contaminating substances including cholesterol are removed from the hormone by extraction with petroleum ether (Stage B). The alcoholic solution of the hormone is treated by a modified procedure which is described in detail in the paragraph headed Stage C.

Stage A.—Occasionally it has been found that peroxides have developed in the ether used for the extraction of the hormone from

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the aqueous solution of the soaps. That this may cause difficulty in the later steps of purification will be brought out in the discussion of our tables. Though the hormone is extremely sensitive to oxidizing agents, the peroxide in ethyl ether seems to cause little trouble with crude preparations and it is only after or during the purification with petroleum ether that the oxidation assumes any quantitative significance.

The modification of this part of the procedure then is: (1) the use of peroxide-free ether for the extraction of the hormone, and (2) the immediate removal of the ether by distillation as soon as the extraction is finished. The ether is distilled off and to make doubly sure of the absence of peroxides, some of the distillate is tested with aqueous KI for peroxide. Thus far we have found that if peroxide-free ether is used for the extraction and the ether extract distilled promptly after the completion of this step, the KI test is negative. After the distillation of the ether, a small volume of alcohol is added to the oily residue, the alcohol heated to boiling, cooled to -10° , and filtered. The residue is washed from the filter paper back to the original flask with alcohol and the leaching repeated. Three or four repetitions are sufficient to remove all of the hormone from the insoluble lipid material and a total volume (distributed through four or five leachings) of 100 to 150 cc. of alcohol is sufficient to remove the entire activity of the extract of 2 liters of liquor folliculi. The insoluble residue may be discarded as its activity is negligible. It has been our experience that this alcoholic solution is a good storage point; its activity seems to remain undiminished even after long periods of standing.

Stage B (Petroleum Ether Removal of Cholesterol, Etc.)—At this stage one-half or even more of the activity may be lost if the ethyl ether used in the earlier steps contained or was allowed to develop peroxides. In addition to this source of difficulty we have encountered still another. Several of our samples of petroleum ether liberate iodine from aqueous solutions of KI, which leads us to believe that they contain peroxides. It has therefore become our practice to test the petroleum ether as well as the ethyl ether with aqueous potassium iodide immediately before using them in the hormone work. Using tested solvents, we have no difficulties with the petroleum ether purification of the hormone.

If we may interpret the experiments of Glimm and Wadehn

(1926) in the light of our unfortunate experiences with peroxides in ethyl ether and petroleum ether, it seems rather probable that their unaccountable losses of activity were due to oxidation of a similar nature. Certain of our earlier experiments in which loss of potency occurred are being repeated owing to the suspicion that possibly the presence of peroxides in solvents has obscured the true behavior.

The removal of cholesterol with petroleum ether has proved to be very satisfactory in the hands of others, the principle of this separation having been adopted by Slotta (1927). We have altered the procedure only in the concentration of alcohol used. The alcoholic solution prepared in Stage A is diluted with an equal volume of water which makes the concentration of alcohol in the hormone solution about 47 per cent by volume. Five extractions are made with redistilled peroxide-free petroleum ether, one-fourth the volume of the aqueous alcoholic solution of hormone being used each time. As pointed out in our earlier paper the distribution ratio of the hormone is such that very little loss occurs but over 95 per cent of the contaminating substances are removed.

Stage C.—The dilute alcoholic solution of hormone is distilled and the residue leached with peroxide-free redistilled ethyl ether. The ethereal solution is immediately distilled and the residue remaining is taken up in redistilled 95 per cent alcohol.

In our earlier publication the opinion was expressed that the ethereal solution of this step would furnish a good stopping place for the accumulation of a large quantity of material for further chemical work. Our later work has definitely disillusioned us on this point, but mere distillation of the ether and the addition of alcohol solve the difficulty.

Stage D (Aqueous Solutions).—As a result of implantation experiments (Zondek and Aschheim, 1925) Zondek (1927) seems to have expressed the idea to Laqueur that the ovarian hormone should be obtainable in an aqueous solution. Two groups of investigators—the one headed by Zondek and the other by Laqueur—shortly after reported the preparation of aqueous solutions of the ovarian hormone (Folliculin B, Zondek and Brahn (1925); and Menformon, Laqueur, Hart, deJongh, and Wijsenbeek (1926) respectively). Other investigators, Glimm and Wadehn (1926), Dickens, Dodds, and Brinkworth (1927), Slotta (1927), and

Biedl (1927), have subsequently reported the preparation of aqueous solutions.

Two different view-points of aqueous solubility must be considered: (1) the extraction of the hormone from ovaries or other tissue with water, and (2) the actual solubility of the refined product in water.

Laqueur (Laqueur, Hart, deJongh, and Wijsenbeek (1926) and Laqueur, Hart, and deJongh (1927)) has sought to avoid the preliminary extraction with alcohol and has to a certain extent succeeded. It is true that his procedures give active extracts but in our hands the losses in the residual tissue are too great to warrant serious consideration being given the process. This, however, may be due to our inability to follow the rather sketchy details of the description, or to Laqueur's willingness to sacrifice yield to secure speed and a high degree of purity of the final product.

Dickens, Dodds, and Brinkworth (1927) have attempted to shorten and improve the extraction by applying the picric acid process used with insulin. However, Laqueur (Laqueur, Hart, and deJongh, 1927) is unable to agree with their claim that their procedure leads to a good product which is water-soluble.

The true aqueous solubility can scarcely be doubted any longer. Both Zondek and Laqueur have adduced evidence that a rather refined product (Laqueur (1927), 0.001 to 0.002 mg. per 1 rat unit) forms a perfectly clear transparent aqueous solution, and the latter has found that the hormone diffuses through parchment and collodion. We have rather hesitated to adopt this view because of the known influence of one substance upon the solubility of another but since our recent success in reducing the rat unit to considerably below 0.001 mg. and thus eliminating much of the contaminating material, we are willing to subscribe to the possibility of aqueous solubility of the hormone. However, we cannot agree with Laqueur's statement that, as the hormone is purified, it loses its solubility in lipid solvents. Certainly our best preparations are still soluble in ether and alcohol. We believe that the question of solubility is so connected with the nature of the contaminating substances that authoritative information must await the actual isolation of the chemical substance.

In view of the recently published data regarding potency (Laqueur, 0.001 to 0.002 mg. per 1 rat unit) solubility in water may be

an exceedingly confusing expression. The solubility in this case measured in physiological activity may be remarkably great, yet in the usual chemical terms exceedingly minute. For example, 10 rat units per 1 cc. of the product just referred to are 0.01 mg. per 1 cc. or 1 mg. per 100 cc., which is little greater than the solubility of many of the salts used in quantitative analytical chemistry.

Preparation of Aqueous Solutions.

After the final alcoholic solution of the hormone is obtained, it is merely diluted with an appropriate volume of water and the alcohol distilled off. Upon cooling, the solution becomes opalescent. A drop of dilute HCl is added to facilitate the flocking of the colloidal impurities and the solution kept in the refrigerator (1°) for a few hours. After filtration through a thick layer of asbestos, the filtrate should be indistinguishable in appearance from distilled water. The considerable portion of the active material which remains on the filter may be recovered by extraction with a small volume of alcohol followed by a repetition of the transference to aqueous solution as just described.

In summarizing the additions to our procedure, attention should again be called to the necessity of using solvents free from peroxides and to the importance of avoiding the storage of the hormone in solvents which may develop peroxides. The conversion of an alcoholic solution of the hormone into a crystal-clear aqueous solution is a simple matter.

DISCUSSION.

The stability of preparations of a physiologically active substance is of importance to the chemist engaged in its isolation and to the clinician who may employ it in the treatment of his patients. Some of our extracts have been kept for a sufficient period of time to warrant a brief discussion.

One of our best extracts was dissolved in ethyl alcohol in 1923. Subsequently, aliquots have been taken for assaying. Occasional tests have shown that the potency has not diminished during the $4\frac{1}{2}$ years of storage in the alcoholic solution.

A crude preparation dissolved in ethyl ether has been stored for about 2 years without any diminution of potency. This is a strik-

ing contrast to the behavior of ethereal solutions of purified preparations which might lead one to suspect the presence of a protective complex in the crude extract.

Properly prepared aqueous solutions seem to retain their potency unimpaired for long periods of time. Our oldest aqueous preparation which was made in December, 1925, is still as active as it was at that time. Another extract made in this laboratory in June, 1926, has retained its full potency whereas an experimental commercial extract has lost about 35 per cent of its strength.

A petroleum ether solution prepared 3 years ago has lost four-fifths of its potency.

Turning our attention to the stability of the highly purified preparations, we may say that both aqueous and alcoholic solutions seem to retain their potency over long periods of time under proper conditions. The most essential condition for stability is the absence of oxidizing agents. Giesy (1920) has already called attention to the destruction of the hormone by a variety of oxidizing agents such as potassium permanganate and bromine. We can add an illustrative experiment in which an unsuspected source of oxidation caused us a great deal of trouble.

If a few cc. of redistilled ethyl ether are added to a tube and the ether allowed to evaporate, a scarcely visible droplet or two may remain. If a highly purified aqueous solution of the hormone is added, a few days suffice to effect a remarkable diminution of potency. In one experiment the residue from 3 cc. of ethyl ether destroyed 5 rat units, in another the residue from 6 cc. destroyed over 13 units. These values must not be regarded as the extent of destruction possible with the volumes of ether used but rather the point at which the experiment was terminated. In the second experiment a volume equivalent to 13 rat units of the original solution was insufficient to produce estrus in a spayed rat; a larger volume was not injected.

Semiquantitative experiments have shown that the residues or the higher boiling fractions of ethyl ether and petroleum ether contain most of the peroxides. Consequently it is conceivable that the use of volatile solvents containing peroxides for the extraction of the hormone may lead to a concentration of the peroxides in the residue containing the active material when the solvent

is distilled off, which may lead to serious losses of the hormone during subsequent purification.

The data of Table I are cited to show how the peroxides may interfere in the further steps of purification. The crude preparations stored as ethereal solutions seemed to possess about the activity expected from the volumes of liquor folliculi extracted. However, when an attempt was made to apply the petroleum ether-

TABLE I.

Effect of Organic Peroxides. Recovery of Activity after Petroleum Ether Extraction of Alcoholic Solution of Hormone.

Preparation No.	No. of petroleum ether extractions.	Rat units.		Recovery.	Remarks.
		Before petroleum ether extraction.	After petroleum ether extraction.		
				<i>per cent</i>	
20	6	870	650	74	
21	6	1260	630	50	
22	6	840	310	37	Conducted in refrigerator.
230	4	200	126	63	Used specially purified petroleum ether.
241 e	5	550	370	67	
25	5	500	330	66	
26	5	400	350	87	Same ethereal solution but distribution of No. 126 carried out 1 year later than No. 26.
126	5	575	115	20	
30	4	32	30	94	
301 a	5	1295	720	56	Distribution of No. 30 carried out after ether solution had been kept 5 mos.
Average recovery with Nos. 26 and 30 omitted.....				54	

70 per cent alcohol distribution for purification, unaccountable losses of potency occurred. In working up the details of the process originally, we were pleased to find that a very advantageous increase of purity could be attained with practically no loss of activity. Upon further application of the procedure losses of such a magnitude were encountered that the method was of no service. After some casting about for a possible cause, our suspicion fell

upon the solvents, some of which upon examination were found to contain peroxides. The explanation now seems to be fairly clear. For instance, Preparation 26 (Table I) retained its potency in ethereal solution unimpaired for 1 year. When it was still fresh and presumably no peroxide was present, it was purified with a recovery of 87 per cent of the rat units. 1 year later a similar number of rat units (Preparation 126) was purified by the same process but in this case only 20 per cent of the active material was

TABLE II.

Effect of Antioxidants. Recovery of Activity after Petroleum Ether Extraction of Alcoholic Solution of Hormone.

Preparation No.	No. of petroleum ether extractions.	Rat units.		Remarks.
		Before petroleum ether extraction.	Recovered after petroleum ether extraction.	
25 e	4	150	120	Small amount cod liver oil, soap, and Na_2SO_3 .
26 c	4	88	88	5 mg. catechol.
26 f	5	44	44	Few mg. hydroquinone.
30 b	8	45	37	Few mg. catechol added with each volume of petroleum ether.
30 c	8	45	37	Few mg. formic acid added with each volume of petroleum ether.
30 d	8	45	37	Few mg. NaHSO_3 added with each volume of petroleum ether.
30 f	8	45	32	Few mg. hydroquinone added with each volume of petroleum ether.
37	5	1280	1200	Shook ether solution with alkaline glucose.

recovered and even this amount decreased rapidly during storage of the purified preparation. The pair, Preparations 30 and 301 a, bring out the same point but in a less striking degree.

Casting about for some method of saving the hormone of our stock solutions which contained peroxide, we tried the use of antioxidants (Moureu and Dufraisse, 1927) without striking success. Sometimes (Table II) an antioxidant appeared to be effective but subsequent trial gave a less favorable result. The effect of a strong reducing agent was also tried with Preparation 37. The

ethereal solution which gave a strong test for peroxides was shaken with 2 portions of an alkaline (NaOH) solution of glucose. Upon subsequently testing for peroxides, their presence could not be detected. The recovery of 94 per cent of the potency of this preparation after purification is regarded as satisfactory.

In Table III we present data upon the recovery of potency when due care was taken to exclude peroxides from the solvents used in the purification. Such a procedure is feasible and seems to be

TABLE III.

Peroxides Excluded. Recovery of Activity after Petroleum Ether Extraction of Alcoholic Solution (50 Per Cent) of Hormone.

Preparation No.	No. of petroleum ether extractions.	Rat units.		Recovery.	Remarks.
		Before petroleum ether extraction.	After petroleum ether extraction.		
				<i>per cent</i>	
31	5	400	355	89	Diminished illumination.
32	5	686	528	77	" "
33	5	480	480	100	" "
35		2000	1666	83	
341 f	5	1690	1500	89	Ordinary daylight.
36	5	475	475	100	" "
361 b	5	2460	2460	100	" "
37	5	1280	1200	94	" "
38	5	1000	1200	120*	" "
381 c	5	1000	1200	120*	" "
39	5	2080	2000	96	" "
Average.....				92	

* Omitted from the average.

entirely satisfactory. The average recovery obtained in Table I is 54 per cent as contrasted with the recovery of 92 per cent in Table III. A word might be said about the omission of Preparations 38 and 381c from the calculation of the average. A recovery of 120 per cent of the potency is scarcely to be expected but, when one considers the uncertainty of the biological assay, it is not surprising. Some of our lower values, as 77 per cent in Preparation 32, may likewise be due to errors of testing. If we should

admit this and trust that the plus and minus errors compensate, then our average recovery becomes 97 per cent.

In the light of our experimental misfortunes due to the presence of peroxides in solvents, we want to reiterate the warnings of Rosenheim (1927) and Holm (1928). Though their papers dealt with the possibility of destroying vitamins by oxidation with peroxides of the solvents, the warning should be extended to include all biological substances of such extreme activity that they

TABLE IV.
Preparation of Aqueous Solution of the Ovarian Hormone.

Preparation No.	Rat units in alcohol solution.	Rat units in aqueous solution.	Rat units in aqueous solution.	Rat units per 1 cc.	Remarks.
			<i>per cent</i>		
31 y	150	132	88	2	Water-clear.
312 e	1240	1025	83	25	" Two aqueous extractions.
331 c	480	302	63	7	Water-clear.
34 f	1500	704	47	22	Slight opalescence.
35	1666	1320	79	16	Water-clear.
36 c	475	394	83	12	" Two aqueous extractions.
361 c	2460	1475	60	24	
360 c	1680	1450	86	42	1 rat unit = 0.0033 mg.*
37 c	960	480	50	10	Water-clear. Two aqueous extractions.
38 c	1200	940	78	27	Opalescent.
381 c	1200	937	78	14	
382 d	1330	1132	85	33	1 rat unit = 0.0029 mg.
43	2500	1640	65	44	Combined residues.

* Subsequently purified to over 1000 rat units per 1 mg.

are measured in physiological rather than metric units. The loss of potency of 1000 rat units is actually the oxidation of less than 1 mg. of the hormone.

Preparation of Aqueous Solutions.

Our early doubts about the aqueous solubility of the hormone were really based upon two points: (1) the incompleteness of the extraction from tissues without the use of organic solvents; (2)

the presence of a faint turbidity in aqueous solutions of our purest preparations. The first point is still of importance but the second is no longer of consequence. Adequate filtration will remove the turbidity leaving a clear, colorless, sparkling solution which possesses potency.

By the principles outlined in an earlier section of this paper, we have prepared a large number of clear aqueous solutions. In no instance, however, have we obtained the full number of rat units in the filtrate, the residue on the filter always containing some active material. Preparation 43, Table IV, represents a solution prepared from the combined insoluble residues of earlier preparations. By working over the precipitates practically all of the potent material may be transferred to a water-soluble form.

We have altered the procedure for the preparation of aqueous solution so frequently that a definite set of conditions cannot be described. It has seemed that the actual recovery of rat units in the aqueous solution is highest when the hormone in a solvent miscible with water is added to water, and the organic solvent distilled off. The quantity of water used has varied. For example, the volume of the filtrate in Preparation 360c was 35 cc. and in Preparation 37c, 48 cc.; the recovery in the first case was 86 per cent but only 50 per cent in the latter.

SUMMARY.

1. Attention is called to the possibility of the destruction of the ovarian hormone by oxidation by the peroxides present in ethyl ether and petroleum ether.
2. The preparation of aqueous solutions of the ovarian hormone is described.

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STUDIES ON BLOOD CELL METABOLISM.

II. THE EFFECT OF METHYLENE BLUE AND OTHER DYES UPON THE GLYCOLYSIS AND LACTIC ACID FORMATION OF MAMMALIAN AND AVIAN ERYTHROCYTES.

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In the preceding paper (1) we have shown that the oxygen consumption of mammalian erythrocytes is enormously accelerated in the presence of minute amounts of methylene blue and certain other dyes. This effect upon the respiration of the mammalian cells differs from the effect of methylene blue upon the erythrocytes of avian blood. In the present paper we present the results of a continuation of this study; namely, a comparison of the influence of methylene blue upon glycolysis and lactic acid formation in mammalian erythrocytes, and in the actively respiring cells of avian blood.

The extensive literature upon glycolysis in the blood has been reviewed by other observers. Lepine (2) first introduced the term and showed that the glycolytic process in blood required the presence of a ferment. Since glycolysis did not occur in serum he stated that it was in some way connected with the blood cells and suggested that the ferment was located in the leucocytes. The important work of L  vene and Meyer (3) demonstrated the r  le of leucocytes in glycolysis. Rona and Arnheim (4), as well as others, have shown that glycolysis is effected by erythrocytes as well.

The studies of recent years have indicated that the disappearance of glucose from the blood (and other tissues) occurs in two different ways. In the first, an actual oxidation with considerable liberation of energy occurs; the second is effected by a splitting of the hexose molecule into 2 molecules of lactic acid, but without a

coincident utilization of oxygen. The latter process has rather striking analogy to the fermentation of yeast cells. It is possible, indeed probable, that some of the sugar first converted into lactic acid is further broken up by oxidation, in which case it falls in the first category.

In order to determine the relative amounts of glucose which disappear by these two paths, a knowledge of the amount of lactic acid produced is required as well as of the amount of glucose which is lost. Since 2 mols of lactic acid are formed from the glycolysis of each mol of sugar, the glucose which is split up in excess of this simple proportion must then disappear by the other route. It is convenient to express this relation by means of what we may call the *glycolytic quotient*, as follows:

$$\frac{\text{Millimols lactic acid produced}}{2 \times \text{millimols glucose degraded}}$$

When this quotient is unity, it indicates that all of the glucose has been converted into lactic acid. When the ratio is less than unity, the fraction expresses the proportion of the total glucose converted to lactic acid, the remainder having disappeared by another channel or by further splitting of lactic acid. A study of this *glycolytic quotient* as found in adult mammalian erythrocytes in blood prepared and freed from leucocytes by the methods detailed in Paper I (1), and incubated as previously described, demonstrated the fact (Tables I and II) first predicted by Claude Bernard¹ that the sugar loss can usually be accounted for in very

¹ It is of interest that the relation between the disappearance of sugar and the formation of lactic acid was first postulated by Claude Bernard who considered that it was a fermentation.

"Ce ferment lactique se rencontre dans le sang, dans les muscles, dans le foie lui-même; car, j'ai constaté que les muscles et divers tissus ne deviennent acides après la mort qu'autant qu'ils renferment du sucre ou de la matière glycogène qui subit très-rapidement une fermentation lactique. J'ai reconnu autrefois [Cours de physiologie, au Collège de France, t. I, pp. 379-392, 1855] cette fermentation lactique de la matière glycogène, d'abord dans les muscles du fœtus, où elle présente son *summum* d'intensité; je l'ai constatée plus tard chez l'homme et les animaux adultes. J'ai vu également la fermentation lactique dans le sang. Quand on prend sur un animal qu'on vient de sacrifier le sang le plus sucré, celui des veines sus-hépatiques, on constate que sous l'influence d'une chaleur modérée

large part by new formation of lactic acid. This glycolysis, which requires no oxygen, is in harmony with the observation that adult mammalian erythrocytes have a scarcely measurable oxygen consumption. The carbohydrate metabolism of the mammalian erythrocyte thus appears to occur without oxygen under normal conditions. In contrast to the *glycolytic quotient* of mammalian blood, that of avian blood is always less than 1, indicating that some oxidative process plays a share in the fate of the sugar which disappears, a fact in agreement with the active oxygen consumption found in nucleated red blood cells.

Action of Methylene Blue and Similar Dyes upon the Glycolysis and Lactic Acid Production of Erythrocytes.

The investigations of Loeb (5) and of Melvin (6) showed that ox and sheep blood under aerobic conditions have very slight glycolysis *in vitro*. A similar behavior on the part of avian blood was later shown by Rüter (7). Glycolysis is more marked in the blood of the dog and of man.

(a) *Mammalian Blood*.—The addition of methylene blue² produces a lowering of the *glycolytic quotient* in mammalian blood. It increases the amount of sugar destruction, but its chief effect

le sucre disparaît du sang en lui donnant une réaction acide, au lieu de la réaction alcaline normale. . . . J'admets donc, quant à moi, que la destruction du sucre a lieu par *fermentation* et non par l'influence directe des alcalis du sang, qui favorisent seulement cette réaction. . . . Si l'on prend deux parties égales du même sang sucré et si l'on fait cuire l'une d'elles de façon à coaguler ou à détruire le ferment lactique, on voit le sang rester alcalin et le sucre persister pendant très-longtemps dans la liqueur." (Bernard, C., Leçons sur le diabète, Paris, 1877, 328.)

² The concentration of methylene blue and of the other dyes used, as in the previous communication, was 0.005 per cent unless otherwise indicated. The same specimens of dyes were employed. Blood sugar determinations were made by Benedict's modification of the Folin-Wu technique. The lactic acid was determined by the modification of Clausen's method introduced by Friedemann, Cotonio, and Shaffer. For each experiment 10 cc. of blood were used. Triplicate blood sugar estimations and duplicate lactic acid estimations were made. The limits of error of the analytic procedures are estimated to be per liter 0.2 mm for lactic acid and 0.25 mm for sugar, under the conditions of these experiments.

is to lessen the coincident lactic acid production (Table I).³ We interpret the meaning of these results to be that methylene blue causes a shift of the course of carbohydrate catabolism away from the (anaerobic) formation of lactic acid and toward the oxidative

TABLE I.

Mammalian Erythrocytes. Comparative Study of Glycolysis and Lactic Acid Formation as Influenced by the Oxidative Action of Methylene Blue (M.B.) (0.005 Per Cent Concentration).

Experiment No.		Blood glucose, mm per l.			Lactic acid, mm per l.			Calculated glycolysis in blood with M. B. mm per l.	Oxidation by M. B., calculated as glucose. mm per l.	Glycolytic quotient.
		Before incubation.	After 3 hrs. incubation.	Decrease.	Before incubation.	After 3 hrs. incubation.	Increase.			
1	Dog blood.									
	Control.....	3.96	2.19	1.77	2.19	5.73	3.54			1.00
	Added M.B.....		2.01	1.95		4.47	2.28	1.14	0.81	0.58
2	Control.....	4.50	2.00	2.50	3.87	8.66	4.79			0.96
	Added M.B.....		1.61	2.89		6.00	2.13	1.12	1.77	0.37
3*	Human blood.									
	Control.....	4.28	2.03	2.25	1.92	6.03	4.11			0.91
	Added M.B.....		0.94	3.34		5.00	3.08	1.69	1.65	0.46
4*	Control.....	4.22	2.12	2.10	2.73	6.90	4.17			0.99
	Added M.B.....		1.38	2.84		6.31	3.58	1.80	1.04	0.63
5*	Control.....	5.98	3.41	2.57	2.71	7.81	5.10			0.99
	Added M.B.....		2.02	3.96		7.77	5.06	2.55	1.41	0.64

* The blood used in Experiment 3 was from a patient with heart disease, in Experiment 4 from a patient with polycythemia, and in Experiment 5 from a patient with hyperthyroidism.

path. The action of methylene blue is most striking in the experiments upon blood with low glycolytic power (Table II). In these

³ For example, in Table I, Experiment 1, the *glycolytic quotient* reached unity in the control sample, indicating that no oxidation had taken place. In the sample containing methylene blue, however, the *glycolytic quotient* was reduced to 0.58. In this case the lactic acid increase was 2.28 millimols, an amount corresponding to a sugar decrease of 1.14 millimols. As the glucose decrease found however was 1.95 millimols, we may conclude

experiments the amount of sugar which disappears in the presence of methylene blue is quite as large as it is in control specimens, which contain no dye, but there is practically no new formation of lactic acid.⁴

(b) *Avian Blood*.—The *glycolytic quotient* in the case of avian blood is in general lower than in the case of mammalian blood, a fact in accord with its higher respiratory metabolism. The experiments on chicken blood, however, examined twice from different birds showed neither disappearance of glucose nor methylene blue action (Table II). It is stated by Bornstein and Ascher (8) that although normal goose blood shows no glycolysis, anemic goose blood has some glycolytic power. In our experiments, no glycolysis was found in blood taken from one goose until after the bird had been bled repeatedly and thus rendered anemic. Material from a second normal goose, however, showed glycolysis even in the first sample taken. The effect of methylene blue upon the *glycolytic quotient* in goose blood is similar to that in mammalian blood.

that 1.14 millimols disappeared by glycolysis and the remainder (0.81 millimols) by oxidation.

Where the *glycolytic quotient* is less than 1 in the control, the corresponding amount of sugar has disappeared by a path other than that of lactic acid formation. The amount of glucose which is consumed coincident with the new lactic acid formation in the methylene blue experiment is then determined on the basis of that produced in the control. This amount deducted from the total amount of glucose which disappears in the methylene blue experiment gives the amount oxidized by reason of the presence of the methylene blue itself. For example, in Table II, Experiment 3, the *glycolytic quotient* is 0.59, indicating that a very large proportion (41 per cent) of the glucose has disappeared by the oxidative path. This must be taken into account in estimating the methylene blue effect, as follows: Since 1.01 millimols of lactic acid were produced in the control during the disappearance of 0.86 millimols of sugar, the production of the 0.27 millimols of lactic acid found in the specimen to which methylene blue was added was associated on this basis with the loss of 0.23 millimols of sugar. The difference between this amount and the total glucose which disappeared (0.89 millimols - 0.23 millimols = 0.66 millimols) is that oxidized by the methylene blue.

⁴ For example, in Experiment 1, Table II, the *glycolytic quotient* of the control blood is equal to unity, while in the methylene blue sample the quotient has fallen to 0.01. This fall is due to the large amount of sugar (0.73 millimols) which has disappeared by oxidation and without coincident increase of lactic acid.

(c) *Effect of Other Dyes.*—In accordance with our findings in the preceding paper on the respiratory metabolism of erythrocytes, the oxidative action of methylene blue upon glucose in the blood is shared by other dyes which Clark (9) has shown to possess similar

TABLE II.

Mammalian and Avian Blood with Low Glycolytic Power. Comparative Study of Glycolysis and Lactic Acid Formation. Oxidative Action of Methylene Blue (M.B.) (0.005 Per Cent Concentration).

Experiment No.		3 hrs. incubation.						Calculated glycolysis in blood with M.B.	Oxidation by M.B., calculated as glucose.	Glycolytic quotient.
		Blood glucose, mm per l.			Lactic acid, mm per l.					
		Before incubation.	After incubation.	Decrease.	Before incubation.	After incubation.	Increase.			
								mm per l.	mm per l.	
1	Ox blood.									
	Control.....	3.61	2.94	0.67	7.50	8.85	1.35	0.10	0.73	1.00
	M.B.....		2.78	0.83		7.52	0.02			0.01
2	Sheep blood.									
	Control.....	2.34	1.60	0.74	4.16	5.58	1.42	0.03	0.79	0.96
	M.B.....		1.52	0.82		4.21	0.05			0.03
3	Goose blood.									
	Control.....	6.39	5.53	0.86	6.18	7.19	1.01	0.23	0.66	0.59
	M.B.....		5.50	0.89		6.45	0.27			0.17
4	Chicken blood.									
	Control.....	14.72	14.68	0.00	3.25	3.27	0.00		No oxidation by M.B.	No glycolysis.
	M.B.....		14.72	0.00		3.26	0.00			

oxidation-reduction potentials. Studies have been made of the effect of toluylene blue, phenol indophenol, and Bindschedler's green (double zinc salt) in equimolar concentration. In each case there is an oxidizing action on the carbohydrates, as the *glycolytic quotient* and calculated glycolysis demonstrate (Table III). The

one exception is phenol indophenol, which in several experiments had little or no demonstrable action (Table III, Experiment 2). The calculated amounts of sugar per liter which have undergone oxidation in the presence of the various dyes, are indicated.

TABLE III.

Mammalian Blood. Comparative Action of Methylene Blue (M.B.), Phenol Indophenol (P.I.), Toluylene Blue (T.B.), and Bindschedler's Green (B.G.) upon Glycolysis and Lactic Acid Formation.

Experiment No.		Blood sugar, mm per l.			Lactic acid, mm per l.			Calculated glycolysis in blood with dyes.	Oxidation by dyes, calcu- lated as glucose.	Glycolytic quotient.
		Before incubation.	After incubation.	Decrease.	Before incubation.	After incubation.	Increase.			
								mm per l.	mm per l.	
1	Human blood.									
	Control.....	4.28	2.03	2.25	1.92	6.03	4.11			0.91
	M.B.....		0.93	3.35		5.00	3.08	1.68	1.67	0.46
	P.I.....		1.53	2.75		6.36	4.44	2.43	0.32	0.81
2	Control.....	4.22	2.12	2.10	2.73	6.90	4.17			0.99
	M.B.....		1.38	2.84		6.31	3.58	1.80	1.04	0.63
	P.I.....		2.18	2.04		6.80	4.07	2.05	0.00	1.00
3	Control.....	5.97	3.40	2.57	2.71	8.00	5.29			1.00
	M.B.....		2.02	3.95		7.96	5.25	2.55	1.40	0.66
	T.B.....		1.90	4.07		7.13	4.42	2.14	1.93	0.54
4	Control.....	5.05	2.65	2.40	2.13	6.26	4.13			0.86
	M.B.....		1.54	3.51		5.84	3.71	2.15	1.36	0.53
	B.G.....		2.13	2.92		5.69	3.56	2.07	0.85	0.61
5	Dog blood.									
	Control.....	4.50	2.00	2.50	3.87	8.66	4.79			0.96
	M.B.....		1.61	2.89		6.00	2.13	1.11	1.78	0.37
	T.B.....		1.56	2.94		7.20	3.33	1.74	1.20	0.57

Effect of Concentration of the Dye.

In harmony with its effect upon the respiratory metabolism, methylene blue exerts its action upon glycolysis and lactic acid formation over a considerable range of concentration. No close relationship was observed between the amount of methylene blue added and the magnitude of the effect on the *glycolytic quotient* or

on the amount of glucose oxidized (Table IV). Methylene blue is active at a concentration as low as 0.0005 per cent. At 0.0001 per cent the oxidizing action is greatly reduced, while it ceases at 0.00005 per cent. The limits of the range of effective concentration of the methylene blue are approximately those found for its effect upon respiration. The wide range of action suggests that the mechanism is one of catalysis. Moreover since methylene blue-methylene white is a highly reversible oxidation-reduction

TABLE IV.
Blood Glycolysis and Lactic Acid Formation. Variation in Concentration of Methylene Blue (M.B.) in Dog Blood.

	Blood glucose, mm per l.			Lactic acid, mm per l.			Calculated glycolysis blood with M.B. mm per l.	Oxidation by M.B. calcu- lated as glucose. mm per l.	Glycolytic quotient.
	Before incubation.	After incubation.	Decrease.	Before incubation.	After incubation.	Increase.			
Control, no M.B.....	4.28	1.97	2.31	2.91	7.52	4.61			1.00
0.1 per cent M.B.....		2.31	1.97		4.82	1.91	0.97	1.00	0.48
0.05 " " "		2.14	2.14		5.20	2.29	1.10	1.14	0.53
0.01 " " "		1.98	2.30		5.00	2.09	1.05	1.25	0.45
0.005 " " "		2.04	2.24		6.00	3.09	1.55	0.69	0.69
0.001 " " "		2.00	2.28		6.00	3.09	1.55	0.73	0.68
0.0005 " " "		1.94	2.34		5.55	2.64	1.32	1.02	0.57
0.0001 " " "		2.00	2.28		6.81	3.90	1.74	0.54	0.86
0.00005 " " "		2.05	2.23		7.35	4.45	2.22	No oxida- tion.	1.00

system, it is comprehensible that the concentration of the dye may have little influence on the oxidative process.

Velocity of Glycolysis and Lactic Acid Formation with Methylene Blue.

The rate of glycolysis appears to differ in the various animal species. Lepine (10) as the result of his experiments on dog blood found the curve of glycolysis to be parabolic in form, while Irving

(11) working with rabbit blood maintains that the rate of glycolysis is linear "over a wide range of values, the rate being about 18 mg. per cent per hour at 38°C." The initial concentrations of glucose and of lactic acid present undoubtedly may have influence upon the velocity of the process.

In some experiments in which the rate of glycolysis only was followed in human blood, the curve was more or less linear in type, while in experiments with dog blood the curve described by

TABLE V.

Rate of Glycolysis and Lactic Acid Formation in Dog Blood with and without Methylene Blue (M.B.), during the First 3 Hours of Incubation (37°).

	Blood sugar, mm per l.			Lactic acid, mm per l.			Glycolytic quotient.
	Before incubation.	After incubation.	Decrease.	Before incubation.	After incubation.	Increase.	
1 hr. incubation, control.....	4.28	3.08	1.20	2.91	5.13	2.22	0.93
1 " " M.B.....		2.70	1.58		4.14	1.23	0.39
After 2 hrs. incubation.							
Control.....		2.43	1.85		6.42	3.51	0.95
Added M.B.....		2.20	2.08		5.46	2.55	0.61
After 3 hrs. incubation.							
Control.....		1.97	2.31		7.56	4.65	1.00
Added M.B.....		1.62	2.66		5.78	2.87	0.53

Lepine was found. Table V presents the data from an experiment with dog blood while Fig. 1 represents the glycolytic curve in the control sample and in the sample containing methylene blue. The curves are similar, the chief difference being that methylene blue has accelerated the process. The lactic acid production in the control blood increases in proportion to the glycolysis as may be seen from the *glycolytic quotients*. In the sample containing methylene blue the *glycolytic quotient* is reduced.

Effect of Temperature.

The velocity of blood glycolysis, being governed by the general laws of enzyme action, is accelerated as the temperature increases until a certain optimum is reached. The action of methylene blue follows the same rule (Table VI). If the blood is subjected to a temperature of 56° for 1 hour in order to destroy the glycolytic ferment, the action of methylene blue is also stopped.

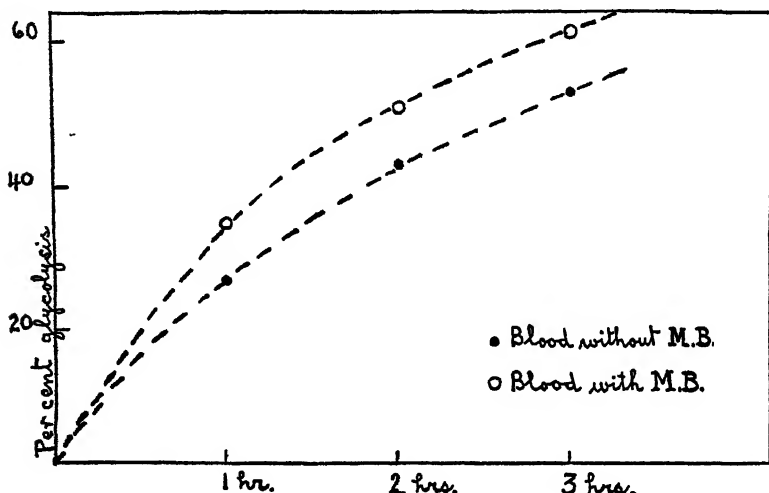


FIG. 1. Dog blood. Curve indicating the rate of glycolysis with and without methylene blue (0.05 per cent).

Action of Cyanides upon Glycolysis and Methylene Blue Oxidation.

According to Warburg's (12) theory, the activation of molecular oxygen by iron is the essential factor in biological oxidations. An important proof is furnished by the effect of cyanides. Warburg (13), from studies on the inhibiting effect of cyanides upon oxidation, asserts that the action is due to a removal of the metal catalyst (iron) to form complex inactive ions (ferro- or ferricyanides). The addition of cyanides on the basis of this theory should not necessarily affect the glycolysis of blood as this seems to require no oxygen. At the concentration of cyanides which we have used (0.001 to 0.002 N, dissolved in 0.9 per cent NaCl) this appears to

be the case. It was deemed important to determine whether the addition of cyanides would prevent the oxidizing action of methylene blue. Potassium cyanide at the concentrations stated above was added to blood and the samples were incubated as usual for 3 hours (38°). The oxidizing action of methylene blue was not at all impaired by the addition of cyanides, but proceeded at the same velocity as in the blood containing methylene blue but no cyanide (Table VII). This oxidation, then, is effected by methyl-

TABLE VI.

Effect of Temperature upon Blood Glycolysis with and without Addition of Methylene Blue (M.B.) (0.005 Per Cent). Defibrinated Dog Blood.
Duration of experiment, 150 minutes.

Experiment No.		Blood glucose.		Glycolysis.
		Before incubation.	After exposure to indicated temperature.	
		gm. per cent	gm. per cent	per cent
1	Kept at ice box temperature (1°).			
	Control.....	0.208	0.207	0
2	Added M.B.....		0.208	0
	At room temperature (22°).			
3	Control.....		0.189	9.1
	Added M.B.....		0.173	16.8
4	At 37° (incubator).			
	Control.....		0.176	15.5
5	Added M.B.....		0.145	30.3
6	Kept 1 hr. at 56°, then placed in incubator (37°).			
	Control.....		0.206	0
7	Added M.B.....		0.206	0

ene blue in atmospheric air in the presence of cyanides and hence presumably without the catalytic action of iron. Thunberg (14) found that the addition of cyanide had no effect on the oxidation of succinic acid to fumaric acid in the presence of methylene blue in muscle preparations under strict anaerobiosis. The oxidizing action of methylene blue was inhibited by cyanide, however, when the experiment was carried on in atmospheric air. Warburg considered that this experiment corroborated his theory in that the

succinic acid and washed muscle in the presence of cyanide form a system which does not take up oxygen. In the glycolysis of blood, however, the oxidation of some intermediary carbohydrate seems to occur with methylene blue in the presence of air without the aid of the iron catalyst. The fact that the oxidizing action of methylene blue upon glucose is not disturbed by the presence of cyanides is in accordance with the lack of influence of cyanides upon methylene blue oxidation noted in the preceding paper.

TABLE VII.

Action of Potassium Cyanide upon Blood Glycolysis and Lactic Acid Formation, with and without Methylene Blue (M.B.) (0.005 Per Cent). Defibrinated Human Blood. Incubation at 37° for 3 Hours.

	Blood glucose, mm per l.			Lactic acid, mm per l.			Calculated glycolysis in blood with M.B. mm per l.	Oxidation by M.B., calcu- lated as glucose. mm per l.	Glycolytic quotient.
	Before incubation.	After incubation.	Decrease.	Before incubation.	After incubation.	Increase.			
Control.....	3.38	1.33	2.05	3.21	6.15	2.94			0.72
Added M.B.....		1.27	2.11		4.92	1.71	1.19	0.92	0.40
“ 0.001 N KCN.....		1.39	1.99		7.11	3.90			0.98
“ 0.001 “ “ +									
M.B.....		1.39	1.99		4.89	1.61	0.82	1.17	0.40
Added 0.002 N KCN.....		1.39	1.99		6.33	3.12			0.78
“ 0.002 “ “ +									
M.B.....		1.36	2.02		4.81	1.60	0.81	1.21	0.40

Action of Phosphates.

The effect of methylene blue upon blood glycolysis in the presence of added phosphate was studied. Two kinds of experiments were performed. In one Sørensen's phosphate mixture (pH 7.4) and glucose (*d*-glucose Pfanstiehl) were added to blood prepared in the usual way, and the glycolysis and lactic acid formation were determined after 3 hours incubation. In the other, phosphates alone were added and the sugar decrease and lactic acid formation were compared in the samples with and without phosphates and

with and without methylene blue (Table VIII). The addition of phosphates appears to increase the oxidative effect of the dyes upon blood glucose.

TABLE VIII.

Action of Phosphates. Blood Glycolysis and Lactic Acid Formation with and without the Addition of Methylene Blue. Defibrinated Dog Blood.

Experiment No.		Blood glucose, mm per l.			Lactic acid, mm per l.			Calculated glycolysis in blood with M.B.	Oxidation by M.B., calcu- lated as glucose.	Glycolytic quotient.
		Before incubation.	After incubation.	Decrease.	Before incubation.	After incubation.	Increase.			
1	Phosphate mixture (pH 7.4) with glu- cose added.							mm per l.	mm per l.	
	Control.....	12.97	9.54	3.43	2.97	7.06	4.09			0.60
	Added M.B.....		8.72	4.25		4.91	1.94	1.62	2.63	0.23
	" P.I.....		8.13	4.84		6.08	3.11	2.60	2.24	0.32
2	Phosphates and glu- cose added.									
	Control.....	16.33	13.54	2.79	5.92	10.64	4.72			0.81
	Added T.B.....		11.81	4.52		8.51	2.59	1.53	2.99	0.29
3	No phosphate added.									
	Control.....	3.38	1.33	2.05	3.21	6.15	2.94			0.72
	Added M.B.....		1.27	2.11		4.92	1.71	1.19	0.92	0.40
4	Phosphate mixture (pH 7.4) added.									
	Control.....		1.22	2.16		6.49	3.28			0.76
	Added M.B.....		1.27	2.11		4.39	1.18	0.78	1.33	0.28

M.B. = methylene blue.

P.I. = phenol indophenol.

T.B. = toluylene blue.

Integrity of Red Blood Cells in Relation to Glycolysis and Methylene Blue Action.

Many authors have observed that the glycolytic process seems closely related to the integrity of the cell surface. Loeb (5) suggested that glycolysis is an intracorpuseular process. Macleod

(15) repeated the suggestion, which correlates his observation that glycolysis is diminished in blood cells washed with saline solution, with the observations of Rona and Döblin (16) that washing rendered the corpuscles incapable of absorbing sugar. We have repeated Macleod's (15) experiments in order to compare glycolysis under such conditions with the oxidative action of methylene blue. The red cells were washed with Locke's mammalian solution instead of saline. It was found that washing of the blood cells decidedly impairs both normal glycolysis and methylene blue action (Table IX).

TABLE IX.

Effect of Alteration or Destruction of Erythrocyte Surface upon Glycolysis with and without Methylene Blue (M.B.). (Washing of Red Blood Cells and Hemolysis.)

Experiment No.		Blood glucose, mm per l.		Glycolysis.
		Before incubation.	After incubation (3 hrs.).	
1	Control blood.....	6.39	5.00	per cent 21.7
	Added M.B.....		4.36	31.7
	Blood washed twice with Locke's solution.....	11.00	10.10	8.2
	Added M.B.....		9.45	14.0
2	Control blood.....	5.55	2.00	64.0
	Added M.B.....		1.55	72.0
	Washed blood cells.....	5.05	3.33	34.0
	Added M.B.....		2.27	55.0
3	Hemolysis.			
	Ox blood hemolyzed with water, control.....	10.00	10.00	0
	Added M.B.....		9.94	0

Rona and Arnheim (4) in 1913 showed that hemolysis with distilled water stops glycolysis. These authors hemolyzed the blood with large amounts of water (20 cc. of blood were mixed with 180 cc. of water). We have compared the glycolysis in normal human blood with that of blood hemolyzed in two ways: (a) repeated freezing and thawing, and (b) hemolysis with distilled water. In the latter case the serum was removed by strong centrifugation and then replaced by an equivalent amount of water.

Glycolysis was impaired in the hemolyzed samples, but not stopped, as the following example shows:

Human blood.	Glycolysis. per cent
(a) Glycolysis after 3 hrs., 37°. Control.....	52
(b) Hemolysis by freezing and thawing.....	36
(c) Hemolysis with distilled water.....	18

In order to observe the action of methylene blue in a sample of blood in which glycolysis was entirely lacking, ox blood was employed. Here (Table IX, Experiment 3) hemolysis with distilled water stopped glycolysis, and methylene blue as well had no action.

TABLE X.

Effect of Hemolysis by Freezing and Thawing upon Defibrinated Goose Blood.

		Glucose, mm per l.		Glycolysis. per cent
		Before incuba- tion.	After incuba- tion.	
Sample A, clear supernatant fluid.	Control.	5.25	5.22	0
	Added methylene blue.		5.25	0
Sample B, thick suspension from bottom.	Control.	4.25	3.85	9.4
	Added methylene blue.		3.33	21.6

Warburg (17) in his studies on the respiration of goose erythrocytes produced hemolysis by repeated freezing and thawing. After prolonged centrifuging of this cytolyzed cell suspension at high speed two layers were formed—the upper containing the hemoglobin and fluid protoplasm, and the lower the solid cell particles, chiefly the nuclei. Respiration took place entirely in this lower layer. We have used Warburg's method to study glycolysis and methylene blue action in anemic goose blood hemolyzed by freezing and thawing. The clear supernatant fluid showed neither glycolysis nor oxidation by methylene blue, while the thick bottom layer showed both normal glycolysis and an increase of this process in the presence of methylene blue. The results of one such experiment are shown in Table X.

Glycolysis and the action of methylene blue therefore seem to be intimately connected with the integrity of the cell structures. When the surface is damaged or destroyed the glycolytic process is slowed or ceases altogether, and with it, the oxidizing action of methylene blue.

Action of Methylene Blue upon Blood Serum and Glucose Solutions.

It has been known for a long time that glucose in alkaline solution can be oxidized by methylene blue. Spoehr (18) observed the

TABLE XI.

Action of Methylene Blue upon Glucose Solutions in Sørensen's Phosphate Mixtures (M/15) at pH 7.40 after Different Times of Incubation (37°).

	Glucose, mm per l.		Observations.
	Before incubation.	After incubation.	
4 hrs. incubation.....	9.26	9.23	No oxidation.
6 " "	8.26	8.26	" "
12 " "	10.91	10.94	" "

TABLE XII.

Action of Methylene Blue upon Glucose and Lactic Acid Content of Blood Serum. Incubation at 37° for 3 Hours.

	Glucose, mm per l.			Lactic acid, mm per l.		
	Before incubation.	After incubation.	Decrease.	Before incubation.	After incubation.	Increase.
Control.....	8.77	8.72	0	5.33	5.33	0
Added methylene blue.....		8.77	0		5.22	0

oxidation of sugar solutions with methylene blue in the presence of disodium phosphate in a continuous stream of air. The catalysis with methylene blue alone occurred only in alkaline solution, disodium phosphate being sufficiently alkaline for this purpose. It could be increased threefold when iron was added, which led him to assume that iron acted as a catalyst. The process described in the present paper is not related to Spoehr's observations as is indicated by the following experiments. Sørensen's phosphate mixture at pH 7.4, containing glucose, was incubated

at 37° for different lengths of time (4, 6, and 12 hours), methylene blue being added in the same concentration as was used in the experiments with blood. The amount of glucose after incubation was unchanged (see Table XI).

The action of methylene blue on serum containing glucose and lactic acid was then examined. Samples of serum were incubated for 3 hours at 37° with and without methylene blue. The glucose and the lactic acid remained unchanged (see Table XII). We conclude that methylene blue has no action upon glucose or lactic acid in the absence of the cells under these conditions.

Effect of Anaerobiosis in Relation to Glycolysis and Methylene Blue Action.

All of the experiments which are reported above have been performed under aerobic conditions. Owing to the method employed for defibrinating the blood and freeing it from leucocytes the material was completely saturated with oxygen just prior to its incubation. In the experiments in which dyes were added, it is true, the oxygen content of the material fell to a low level at the end of the experiment, owing to the increased oxygen consumption of the erythrocytes under such conditions, as was previously reported (1). In the following experiments, a comparison of glycolysis and lactic acid formation was attempted between completely reduced blood and blood which was kept completely and continuously oxygenated. A sample of blood was divided into four flasks, which were arranged in pairs connected with tubing in the thermostat at 38°. In one flask of each set methylene blue was added (0.005 per cent). The stoppers were sealed and a continuous slow stream of nitrogen was passed through one pair of flasks, while oxygen was passed through the other. The flasks were continuously agitated in a shaking device for 3 hours, at the end of which period the glucose and lactic acid contents were redetermined. (Table XIII.)

A constant stream of oxygen passing through blood undergoing glycolysis does not appear to affect this process appreciably as no difference was found between the samples exposed to the gas stream and control samples kept in stoppered flasks. On the other hand a stream of nitrogen which produced relative anaerobiosis increased glycolysis as well as lactic acid formation. In these

TABLE XIII.

Action of Methylene Blue (M.B.) upon Blood Glycolysis and Lactic Acid Formation in Oxygenated and Reduced Blood. Incubation at 38° for 3 Hours.

Experiment No.		Blood glucose, mm per l.			Lactic acid, mm per l.			Calculated glycolysis in blood with M.B.	Oxidation by M.B., calcu- lated as glucose.	Glycolytic quotient.
		Before incubation.	After incubation.	Decrease.	Before incubation.	After incubation.	Increase.			
1	Goose blood.							mm per l.	mm per l.	
	Oxygenated blood.									
	Control.....	8.48	6.11	2.37	5.87	7.07	1.20			0.26
	Added M.B.....		5.34	3.14		6.52	0.65	1.28	1.86	0.10
	Reduced blood.									
	Control.....		4.27	4.21		11.36	5.49			0.65
2	Added M.B.....		4.21	4.27		10.68	4.81	3.70	0.57	0.55
	Oxygenated blood.									
	Control.....	8.72	6.61	2.11	7.44	8.34	0.90			0.21
	Added M.B.....		4.96	3.76		8.03	0.59	1.38	2.38	0.08
	Reduced blood.									
	Control.....		4.90	3.82		12.00	4.56			0.60
3	Added M.B.....		4.87	3.85		11.72	4.28	3.60	0.25	0.55
	Oxygenated blood.									
	Control.....	7.55	6.00	1.55	4.66	5.37	0.71			0.23
	Added M.B.....		4.20	3.35		4.66			3.35	
	Reduced blood.									
	Control.....		4.45	3.10		8.43	3.77			0.61
4	Added M.B.....		3.83	3.72		9.12	4.56	3.74		0.61
	Human blood.									
	Oxygenated blood.									
	Control.....	6.17	3.96	2.21	2.70	7.10	4.40			1.00
	Added M.B.....		2.78	3.39		5.85	3.15	1.58	1.81	0.46
	Reduced blood.									
5	Control.....		3.61	2.56		7.80	5.10			1.00
	Added M.B.....		3.17	3.00		7.80	5.10	2.56	0.44	0.85
	Dog blood.									
	Reduced blood.									
	Control.....	8.16	6.16	2.00	2.78	6.22	3.44			0.85
	Added M.B.....		5.80	2.36		6.45	3.67	2.14	0.22	0.77

experiments it will be seen that the *glycolytic quotient* in the reduced blood, although higher than in the oxygenated blood, never reached unity, a fact which indicates that some oxidative process was going on while the blood was being reduced. It is known that muscle tissue in the presence of oxygen does not show a marked increase in lactic acid as this substance is in part oxidized and in part resynthesized to glycogen; under anaerobic conditions the amount of glucose destroyed corresponds to the lactic acid increase. In the present experiments the lactic acid formed in oxygenated goose blood is so low as to bring the *glycolytic quotient* down to 0.20, while in reduced blood (relative anaerobiosis) there is a rise of the lactic acid content which brings the *glycolytic quotient* up to 0.50 or 0.60. The similarity to the mechanism observed in muscle is evident.

Methylene blue, which produced its usual effect in the presence of oxygen, showed scarcely any oxidizing action upon the reduced blood. In one instance (Experiment 3, Table XIII) where nearly one-half of the glucose was oxidized in the presence of oxygen (no lactic acid increase), none was oxidized in the blood reduced by the nitrogen stream. It seems probable that all of the experiments would have shown similar results had strict anaerobiosis been attained. It is evident however that such a condition was not attained because the nitrogen used, while of high purity, contained on analysis traces of oxygen, which were also found in the blood on analysis at the end of the experiments.

In similar experiments with mammalian blood (Experiments 4 and 5, Table XIII) the flasks containing the samples were kept for 1 hour at 38° in a shaking device in a stream of pure nitrogen.⁵ When the color of the blood indicated complete reduction, methylene blue was added. The flasks were then maintained under the same conditions for 2 or 3 hours. Even with these precautions oxidation by methylene blue was not completely stopped, but it was extremely small.

The failure of methylene blue to produce oxidation in blood undergoing glycolysis in the absence of oxygen is of some significance. A strong argument in favor of Wieland's theory is

⁵ The highly purified nitrogen used in these experiments was prepared in an apparatus to be described shortly by Professor Michaelis to whose kindness we are indebted for the use of this material.

based upon Thunberg's (14) observation that methylene blue oxidizes succinic acid under anaerobic conditions. The presence of the oxyhemoglobin makes strict anaerobiosis difficult in material containing erythrocytes. The question is being studied with reference to the glycolysis produced by leucocytes. Of interest in relation to the effect of methylene blue upon erythrocyte oxidation under anaerobic conditions is the recent communication of Baumberger (19), who found that methylene blue at a concentration of 1 to 1,000,000 had a marked effect in postponing the onset of fatigue in frog muscle under anaerobic conditions. The oxygen equivalent of the methylene blue was far too small to account for the increased output of work produced. The changes in the lactic acid produced were not reported.

DISCUSSION.

It has been shown above that minute quantities of methylene blue in isotonic saline solution, when added to blood undergoing glycolysis, alter the carbohydrate metabolism in such a way that the velocity of sugar transformation is increased, while at the same time less lactic acid is formed. Coincident with this shift in the path of the metabolism there is a marked increase in oxygen consumption and carbon dioxide production, as shown in Paper I. We suggest that methylene blue produces its effect so far as carbohydrate is concerned by increasing the oxidation of some degradation product of glucose. This effect is not produced in blood in which normal glycolysis is absent, nor does it occur either in blood serum or in glucose solutions at pH 7.4. It is therefore not analogous to the oxidation of glucose by methylene blue in alkaline solutions as reported by other observers.

The point in the glycolytic process at which methylene blue exerts its action cannot be determined from our data. The chain of transformations which has been suggested by Shaffer (20) is well known: glucose \rightarrow hexosephosphate \rightarrow glyceric aldehyde \rightarrow methylglyoxal \rightarrow lactic acid.

Of these steps, the transformation of glucose to hexosephosphate, which does not require the presence of oxygen, and the splitting of glyceric aldehyde and methylglyoxal, which are transformed at once to lactic acid, seem to be excluded. Methylene blue has no effect on the oxidation of lactic acid in serum in which there is no

glycolysis. Where glycolysis does not occur in avian blood the addition of methylene blue produces no change in the concentration of lactic acid. Fürth and Lieben (21), however, have shown that lactic acid is consumed by horse erythrocytes, and Ray (22) working, it is true, at the unphysiological pH value of 6.9 has demonstrated the oxidation of lactic acid by dog erythrocytes. One cannot exclude the possibility that the observed methylene blue effect, at least in part, is due to oxidation of lactic acid.

In favor of the possibility that the principal point at which the methylene blue acts is upon the oxidation of hexosephosphate, is the fact that the action of methylene blue is increased by increasing the inorganic phosphate concentration, either alone, or together with added glucose. As to the exact nature of the methylene blue effect little may be said. It is conceivable that it acts as a coenzyme or catalyst, rendering the substrate (hexosephosphate?) more sensitive to the action of molecular oxygen. On the other hand one might consider that methylene blue plays in this system the rôle ascribed to iron in the oxidations produced by Warburg with his charcoal model.

CONCLUSIONS.

The relationship between glycolysis and the oxidative processes in blood cells may be expressed by the ratio

$$\frac{\text{Millimols of lactic acid increase}}{2 \times \text{millimols glucose decrease}}$$

which is termed the *glycolytic quotient*.

Normal adult mammalian red blood cells with almost no oxidative processes show a high *glycolytic quotient*. Nucleated erythrocytes (avian blood cells) with appreciable oxidative processes have a low *glycolytic quotient*.

The addition of methylene blue (or dyes with similar oxidation-reduction potentials) to the blood undergoing glycolysis produces an increased sugar degradation and a diminished formation of lactic acid. This is probably due in large part to the oxidation of some intermediary product. The *glycolytic quotient* is lowered.

The process begins only after the glucose molecule is acted upon by the glycolytic enzyme and is converted into other unstable and oxidizable substances. It is suggested that oxidation may

take place in the early stages of dissociation, before the glucose molecule is split into three carbon chain fragments, probably when the glucose molecule is esterified to hexosediphosphoric acid.

This reaction probably requires the presence of oxygen but the addition of cyanides does not impair the oxidative process. It is suggested that methylene blue acts as a catalyst, rendering the hexosephosphate molecule more sensitive to oxidation by molecular oxygen.

Our thanks are due to Professor Michaelis for his suggestions concerning a number of questions which have arisen in the course of this study.

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THE ELECTROMETRIC TITRATION OF HEMIN AND HEMATIN.

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It was shown 5 years ago (1) that the change from methemoglobin to hemoglobin could be studied by an electrochemical method. The reduction by means of sodium hydrosulfite and the oxidation of the reduced compound (hemoglobin) by ferricyanide were found to involve 1 equivalent. These results have been confirmed and extended in later work (2). An application of the same method to a study of the reduction of alkaline hematin solutions (1) yielded uncertain results which seemed to indicate that 2 equivalents of hydrogen were involved in the reduction of this compound. No definite conclusion could be drawn from the data, however. A continuation of this work has now shown that satisfactory electrochemical titration curves may be obtained *if titanous tartrate is employed as the reducing agent*. The results are very definite and show that the change involves *only 1 equivalent*.

While this work was in progress, two other workers reported results obtained by entirely different methods. These show that the reduction of hematin, like the reduction of methemoglobin, is the change from a ferric to a ferrous compound. The experiments which we now have to report, therefore, serve merely to confirm the recent observations of Haurowitz (3) and those of Hill (4).

Titration of Hemin and Hematin in Borate-Tartrate Buffer Solutions.

In Fig. 1 are shown typical titration curves of hemin solutions with titanous chloride in the presence of tartrate. The crystalline

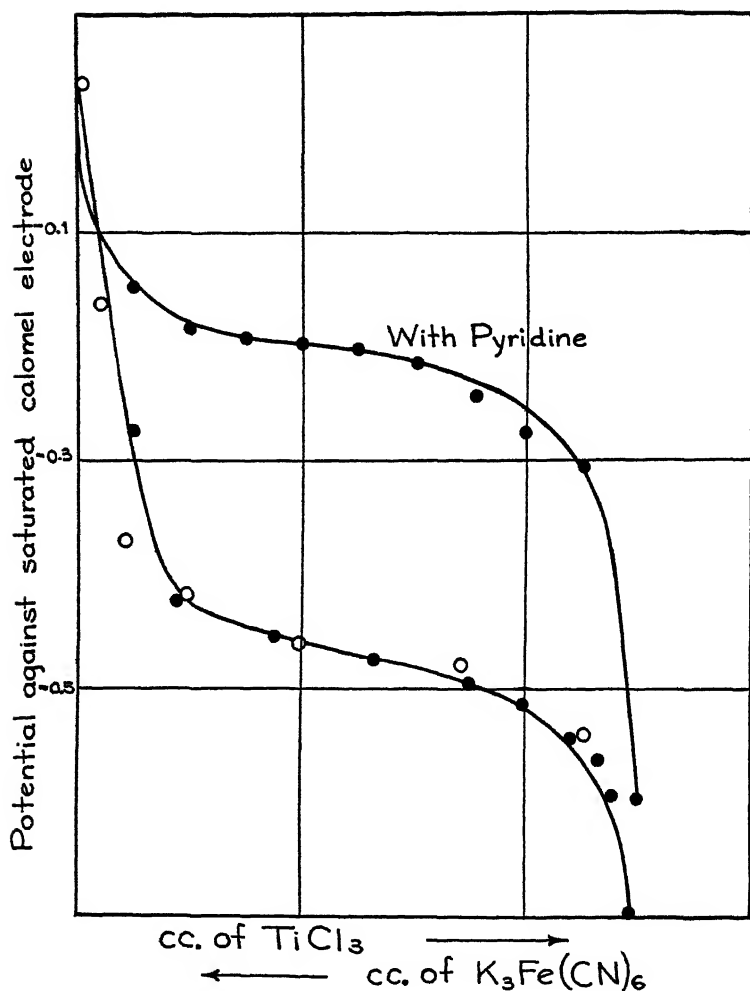


FIG. 1. Electrometric titration of hemin dissolved in borax-tartrate buffer solutions (pH 9.15). Clear circles indicate the points obtained in the back titration with ferrieyanide.

hemin¹ was dissolved in 0.1 M borax either at room temperature or by warming, an equal volume of 0.2 M sodium tartrate was added, and the mixture placed in the usual electrochemical cell. After sweeping out all the air with oxygen-free nitrogen, dilute titanous chloride was added from a burette and the potential of two bright platinum electrodes noted. The potential was measured against a saturated calomel electrode. It was found necessary to use special precautions in purifying the nitrogen since the reduced hematin is extraordinarily sensitive to oxygen. Metallic nickel at a red heat or long trains of ammoniacal cuprous solutions were found to be fairly satisfactory.

The potentials are plotted vertically in Fig. 1 and the increments of reducing or oxidizing agent are plotted horizontally. The back titration with potassium ferricyanide is shown by clear circles. It is evident that the end-points are clearly defined. This is particularly true in the case of the titanous chloride titration in the presence of pyridine which causes a change of the position of the curve because the potential of the pyridine hemochromogen system is more positive than that of the reduced hematin system.

The presence of the tartrate ion in the alkaline buffer solution prevents the precipitation of titanous and titanic hydroxides. Thus, an alkaline complex titanous tartrate is in reality the reducing agent employed but, as solutions of it are very sensitive to oxygen, this indirect method of adding it was finally adopted. The addition of 3 per cent of mannite instead of sodium tartrate will also prevent the precipitation of titanous or titanic hydroxide but the titanous mannite complex does not have a sufficiently low potential to give a sharp end-point. The very dilute titanous chloride used was made by diluting the commercial 20 per cent material with boiled water; it was stored in an atmosphere of nitrogen. It slowly changed and was made up fresh every few days. It was standardized just before or after each titration by titrating electrochemically with potassium ferricyanide.

A few of the many results we have obtained are given in Table I

¹ The hemin used in this work was the α -hemin which was prepared by the usual acetic acid method from horse blood. It was purified by solution in chloroform and pyridine and reprecipitation in acetic acid containing sodium chloride. A few experiments with β -hemin prepared by Mörner's procedure showed that its reduction also required only 1 equivalent.

which is self-explanatory. The numbers in the last column show that within the limits of the experimental error there is 1 mol of titanous chloride used in the reduction and 1 mol of ferricyanide in the reoxidation. The result is independent of the method of

TABLE I.

Determination of Number of Equivalents Involved in Reduction of Hemin or Hematin and Reoxidation of the Reduced Compound.

Experiment No.	pH	Iron compound.	Mols $\times 10^4$.	Reagent employed.	Molality of reagent.	Amount of reagent.	Mols of reagent $\times 10^4$.	Ratio of mols of reagent to mols of compound.
		mg.				cc.		
1	10.26	61.9	0.94	TiCl ₃	0.0174	7.00	1.24	1.3
1 a	10.26	61.9	0.94	K ₃ Fe(CN) ₆	0.0200	4.50	0.90	0.96
2	9.15	12.4	0.19	TiCl ₃	0.0189	0.96	0.18	0.95
2 a	9.15	12.4	0.19	K ₃ Fe(CN) ₆	0.0200	0.89	0.18	0.95
3	9.15	12.4	0.19	TiCl ₃	0.0150	1.35	0.20	1.05
4	9.15	12.4	0.19	"	0.0125	1.95	0.24	1.3
5*	9.09	61.9	0.94	"	0.0167	6.00	1.00	1.06
5 a*	9.09	61.9	0.94	K ₃ Fe(CN) ₆	0.02	5.00	1.00	1.06
6*	9.1	12.4	0.19	TiCl ₃	0.0100	2.00	0.20	1.05
7*	9.1	12.4	0.19	"	0.0111	1.80	0.20	1.05
8	9.15	12.4	0.20	"	0.0140	1.40	0.20	1.0
9	9.15	12.4	0.20	"	0.0125	1.80	0.23	1.15

50 cc. of 0.1 M borax and 0.2 M sodium tartrate were used in each experiment; in those marked with an asterisk (*) pyridine was used also; sodium hydroxide was added in Experiment 1. Experiments 1 to 7 were performed with crystalline α -hemin, Experiment 8 with hematin prepared according to Küster (5), and Experiment 9 with dehydrochlorohemin. In Experiments 1, 5, and 7 the hemin was dissolved at room temperature; in the other experiments the iron compound was dissolved by warming it with the borax solution to 70° for 5 minutes, cooling to 20°, and adding the tartrate. The titrations with ferricyanide (Experiments 1 a, 2 a, 5 a) were performed with the solution reduced with TiCl₃ in the experiments of the same numbers.

dissolving the hemin and independent of the presence of pyridine. Hematin (Experiment 8) and dehydrochlorohemin (Experiment 9) also require 1 equivalent for the reduction. There can be no doubt from these results that the change in each case involves the reduction or the oxidation of the iron atom.

Aside from the information in regard to the number of equivalents involved in the oxidation or reduction, a satisfactory method of studying the iron-porphyrin compounds is of some interest, since it provides another method of characterizing these substances. This is of particular value in the case of the formation of easily dissociable complexes such as are formed between hemin and pyridine. Although the potentials are not as definite or as reproducible as in the case of the quinones, the method is useful even in extremely dilute solutions. For example, we have been able to obtain fairly satisfactory results in solutions of the pyridine-hemin compound which contained only 3×10^{-7} mols of hemin per liter. An electrochemical titration thus promises to be a useful supplement to the spectroscopic method of detecting and studying those iron-porphyrin derivatives which occur widely distributed in nature. We are now engaged in determining the oxidation-reduction potential of all the various iron-porphyrin compounds which can be obtained, including those formed by the decomposition of hemoglobin (α -hemin, β -hemin, "Verdaunung's hematin," etc.) and their combinations with such substances as pyridine and the cyanide ion. The results of this work will be the subject of a later paper.

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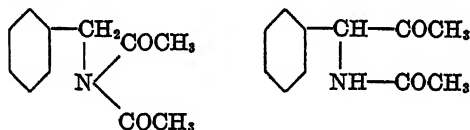
THE ACTION OF ACETIC ANHYDRIDE AND PYRIDINE ON AMINO ACIDS.

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(Received for publication, June 26, 1928.)

In our first communication on this subject,¹ we reported the formation of unexpected derivatives of tyrosine and of phenylaminoacetic acid. In the early experiments with tyrosine, the crude reaction product appeared to have been obtained in a purer condition when the preparation was carried out in the presence of acetone. It therefore seemed possible that acetone took part in the reaction. Two possible structural formulæ were then given for the compound. However, in the experiment with phenylaminoacetic acid there was observed an abundant evolution of carbon dioxide. Hence, for this derivative two alternative formulæ were given.



It was definitely stated that the tyrosine derivative might have the same structure as that of phenylaminoacetic acid. In view of the lack of material and because of pressure of other work, the results have been published in a preliminary way. It was stated that the work on the details of the reaction was being continued.

It has since been found that the compounds described in the first paper are derivatives of acetylaminoacetone.

The constitution of the derivative from phenylaminoacetic acid was the first to have been established. On hydrolysis with hydrochloric acid it was converted into the corresponding hydro-

¹ Levene, P. A., and Steiger, R. E., *J. Biol. Chem.*, 1927, lxxiv, 689.

chloride, which on treatment with alkali in the presence of oxygen of the air yielded a pyrazine. These compounds were identical with the hydrochloride of 1-phenyl-1-aminoacetone and with 3,6-dimethyl-2,5-diphenylpyrazine, both of which have been described previously. Analogous compounds were obtained from the tyrosine derivative.

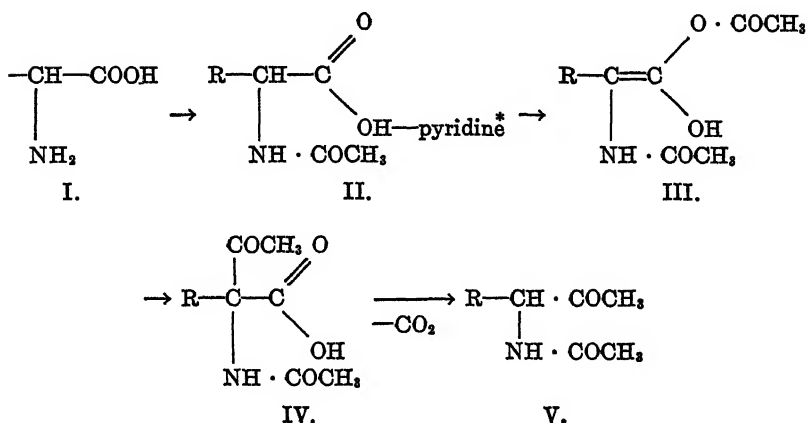
A uniform procedure was then adopted for preparing the acetylaminoketones. It consists of heating the amino acid (0.1 mol) with a mixture of equal weights of acetic anhydride² (100 gm.) and pyridine (100 gm.) in a bath maintained at 90° until the evolution of carbon dioxide has ceased.

This procedure has been applied to phenylaminoacetic acid, phenylalanine, *l*-tyrosine, and phenylmethylaminoacetic acid. The carbon dioxide evolved during the reaction corresponded to 70 per cent of the theory in the case of the first three amino acids. The compounds obtained from *l*-tyrosine and phenylaminoacetic acid were identical with those described in our previous communication. A control experiment was performed in which phenylaminoacetic acid was heated with acetic anhydride in absence of pyridine. No loss of carbon dioxide occurred. In another experiment acetylphenylaminoacetic acid was used instead of the free amino acid, all other conditions being identical. The usual loss of carbon dioxide was observed. However, no carbon dioxide evolution was observed in the case of phenylmethylaminoacetic acid. Here the reaction product was the acetylated amino acid. The experiment is of great significance as it indicates the importance for the reaction of the presence of a mobile hydrogen atom on the carbon atom (2); it is also important as it shows that in the absence of such an atom even a mixed anhydride does not form. Of great importance in connection with this reaction is the fact that from the active tyrosine an inactive aminoketone is formed.

These observations lead us to believe that under the influence of pyridine, enolization occurs which permits the entry of an acetyl group in the molecule on the hydroxyl hydrogen of the carboxyl. The subsequent step consists of a migration of the

² Pure acetic anhydride (b.p. 139.46° under 760 mm.), obtained by a process developed by one of us (S.), was used throughout.

acetyl group to carbon atom (2); this step is then followed by carbon dioxide elimination with the formation of the ketone. The steps may be represented as follows:



(R = C₆H₅; CH₃CO · O · (p) · C₆H₄CH₂; C₆H₅ · CH₂)

Azlacones are probably formed as secondary products of the reaction.

When our work was completed, there appeared an article by Dakin and West,³ who also have prepared acetylated aminoketones by the action of acetic anhydride on amino acids in the presence of pyridine. These authors have applied the method to a larger number of amino acids than we have done and also to other acids.

There is one point in the article of Dakin and West for which, at the present, we can give no explanation; namely, the discrepancy in the melting points of the substances C₁₂H₁₅O₂N obtained on hydrolysis of the O-acetyl-N-acetylaminoketone derived from tyrosine. An error in the introduction of the article of Dakin and West wrongly attributes the discrepancy to the substance C₁₄H₁₇O₄N. The substance of this composition prepared by Dakin and West and the one prepared by us have the same melting point.

* The formation of a pyridine complex of unknown composition is assumed.

³ Dakin, H. D., and West, R., *J. Biol. Chem.*, 1928, lxxviii, 91.

EXPERIMENTAL.

0.1 mol of amino acid was heated with a mixture of pure acetic anhydride (100 gm.) and dry pyridine (100 gm.) in a bath maintained at 90°. The carbon dioxide which escaped from the top of the reflux condenser was washed in a water scrubber and then absorbed in 1.0 N sodium hydroxide. The gas evolution ceased after 2½ hours. Carbon dioxide-free air was used to drive all carbon dioxide from the flask, condenser, and scrubber into the gas absorption apparatus. The carbon dioxide was determined by titration, phenolphthalein being used as an indicator. The reaction mixture was concentrated under reduced pressure to a thick syrup. It was twice reevaporated, each time with 25 cc. of xylene, in order to remove the residual pyridine and acetic anhydride. When the mixture was cooled and left to stand, crystallization took place. Purification of the crude products is best accomplished by crystallization from ketonic solvents (acetone, methylethylketone) despite the difficulties arising from the great solubility of the compounds in such solvents. The products were finally washed on the filter with ether. Further quantities of material were obtained from the mother liquors. When the hydrochlorides of the aminoketones are desired, the crude reaction product can be directly hydrolyzed with hydrochloric acid. Good yields of hydrochlorides are thus obtained. This procedure is more advantageous from a preparative point of view.

1-Phenyl-1-Acetylaminoacetone.—15.2 gm. of phenylaminoacetic acid yielded 19.2 gm. of crude crystalline product. It was purified by crystallization from 10 gm. of acetone. A first batch of crystals (8.3 gm.) melting at 100–101° was obtained.

No. 145.

C₁₁H₁₃O₂N (191.17). Calculated. C 69.08, H 6.85, N 7.33.

Found. " 69.09, " 6.98, " 7.19 (Kjeldahl).

Molecular Weight Determination by Method of Menzies and Wright.—29.0 cc. of acetone (b.p. 56.5°, at 764 mm.); 0.6751 gm. substance; 22.5 mm. elevation on differential thermometer. Found 196. Calculated 191.2.

1-Phenyl-1-Aminoacetone Hydrochloride.—1. The crude crystalline reaction product (18.9 gm.) obtained on heating 19.4 gm. of

acetylphenylaminoacetic acid⁴ with 95 gm. of acetic anhydride and 100 gm. of pyridine was refluxed for an hour with 200 cc. of 5.0 N hydrochloric acid. The dark solution, which had a very displeasing odor, was diluted with 200 cc. of water and 2 gm. of norit were added. When the filtrate was concentrated under reduced pressure, crystallization of the hydrochloride took place. The crystals were filtered off, washed with hydrochloric acid, then with ether, and dried in a vacuum desiccator over phosphorus pentoxide and soda-lime. The product (11.9 gm.) turned red at about 190° and melted around 200° with decomposition.

No. 141.

$C_9H_{12}ONCl$ (185.62). Calculated. N 7.55, Cl 19.10.

Found. " 7.28 (Kjeldahl), Cl 20.37 (Carius).

2. Acid hydrolysis was performed on pure 1-phenyl-1-acetylaminacetone. 7.7 gm. (0.04 mol) were heated for an hour with 100 cc. of 5.0 N hydrochloric acid. The solution was diluted with an equal volume of water and finally evaporated to dryness under reduced pressure. The residue was dissolved in water, the solution decolorized with norit, and after addition of some hydrochloric acid, evaporated to dryness. Drying of the crystals was completed by their standing in a vacuum desiccator over phosphorus pentoxide and soda-lime. The product (6 gm.) turned red at about 190° and melted around 200° with decomposition.

No. 142.

$C_9H_{12}ONCl$ (185.62). Calculated. N 7.55, Cl 19.10.

Found. " 7.28 (Kjeldahl), Cl 20.19 (Carius).

When recrystallized from absolute alcohol, the hydrochloride analyzed as follows:

No. 137.

Calculated. N 7.55, Cl 19.10.

Found. " 7.54 (Dumas), Cl 19.04 (Carius).

It turned red at 200° and melted at 203°. The following data on this compound are recorded in the literature.

⁴ Prepared according to the indications of Knoop, F., and Blanco, J. G., *Z. physiol. Chem.*, 1925, cxlvi, 273.

	Red coloration. °C.	M.p. °C.
Kolb. ⁵	200	208
Gabriel. ⁶	Around 190.	Around 200.
Emde. ⁷	198-199	198-199

3,6-Dimethyl-2,5-Diphenylpyrazine.—1 gm. of 1-phenyl-1-aminoacetone hydrochloride was dissolved in 10 cc. of water. 0.35 cc. of ammonium hydroxide (*d* 0.90) was added. The precipitate which formed was washed with water and dried over phosphorus pentoxide. Yield 0.54 gm. It was dissolved in 4.5 gm. of boiling alcohol to which 1.8 gm. of water were then added. On being cooled crystals separated which were washed with 2.5 cc. of 70 per cent (by weight) alcohol. M.p. 124-125°. Kolb⁸ found 124°, Gabriel,⁹ 125-126°.

No. 140.

C₁₃H₁₁N₂ (260.24). Calculated. C 83.03, H 6.20, N 10.77.
Found. " 82.54, " 6.37 " 10.44 (Dumas).

1-Benzyl-1-Acetylaminoacetone.—16.5 gm. of phenylalanine yielded 21 gm. of crude crystalline product. It was recrystallized from acetone. A first fraction (7.9 gm.), melting at 95-96° was obtained. On recrystallization from acetone, the compound melted at 95-95.5°.

No. 146.

C₁₁H₁₁O₂N (205.19). Calculated. C 70.21, H 7.37, N 6.83.
Found. " 70.56, " 7.72, " 6.71 (Kjeldahl).

1-Benzyl-1-Aminoacetone Hydrochloride.—The crude crystalline reaction product obtained from 16.5 gm. of phenylalanine was refluxed for an hour with 200 cc. of 5.0 N hydrochloric acid. The solution was shaken with 2 gm. of norit, then diluted with 200 cc. of water, and filtered. The filtrate was concentrated under reduced pressure to a thick syrup which had a very unpleasant odor. 10 cc. of xylene were added, and evaporation repeated in order to remove water. Crystallization then took place. This

⁵ Kolb, A., *Ann. Chem.*, 1896, ccxc, 276.

⁶ Gabriel, S., *Ber. chem. Ges.*, 1908, xli, 1152.

⁷ Emde, H., *Arch. Pharm.*, 1909, ccxlvii, 134.

⁸ Kolb, A., *Ann. Chem.*, 1896, ccxc, 277.

⁹ Gabriel, S., *Ber. chem. Ges.*, 1908, xli, 1154.

residue was dissolved in 50 cc. of hot absolute alcohol, the solution filtered hot, and the hydrochloride precipitated by addition of 150 cc. of anhydrous ether. The crystals were filtered off after being cooled in ice, and washed with anhydrous ether. Yield 11.8 gm. M.p. = 123–125°.

No. 144.

$C_{16}H_{14}ONCl$ (199.63).

Calculated. N 7.02, Cl 17.76.

Found. " 6.88 (Kjeldahl), Cl 18.28 (Volhardt).

This product was not further purified.

3,6-Dimethyl-2,5-Dibenzylpyrazine.—2 gm. of 1-benzyl-1-aminoacetone hydrochloride were dissolved in 20 cc. of water. The oil precipitated on addition of 0.7 cc. of ammonium hydroxide (d 0.90) became solid after prolonged stirring. The product was washed with water and dried over phosphorus pentoxide. Yield 1.23 gm. It was dissolved in 4 gm. of boiling alcohol and 1 gm. of water was added; crystallization then set in. The crystals were washed with 50 per cent (by weight) alcohol and recrystallized from absolute alcohol. M.p. = 100–100.5°.

No. 149.

$C_{20}H_{20}N_2$ (288.28). Calculated. C 83.29, H 6.99, N 9.72.

Found. " 83.25, " 7.17, " 9.89 (Dumas).

1-(Acetyl-p-Hydroxybenzyl)-1-Acetylaminoacetone.—37 gm. (0.2 mol) of *l*-tyrosine, 200 gm. of acetic anhydride, and 200 gm. of dry pyridine were heated at 90° for 2½ hours. The solution was concentrated under reduced pressure to a syrup and the evaporation repeated three times, with addition each time of 50 cc. of xylene. The residue, which became solid on being cooled, was dissolved in 40 gm. of pure boiling methylethylketone. The solution was filtered while hot and the crystals deposited under cooling were washed on the filter first with a small amount of ketone, then with anhydrous ether. Yield 29 gm. A second fraction (6.5 gm.) was obtained from the mother liquor. These two fractions were combined and recrystallized from 40 gm. of methylethylketone. 24.1 gm., melting at 121–122.5°, were obtained. When recrystal-

lized twice from double its weight of acetone, a product melting at 123–124° was obtained.

No. 150.

$C_{14}H_{17}O_4N$ (263.22). Calculated. C 63.85, H 6.51, N 5.32.

Found. " 64.21, " 6.76, " 5.19 (Kjeldahl).

This compound was optically inactive.

1-(p-Hydroxybenzyl)-1-Aminoacetone Hydrochloride.—2.7 gm. (0.01 mol) of pure acetylated aminoketone from tyrosine were refluxed for 1 hour with 30 cc. of 5.0 N hydrochloric acid. The solution was then diluted with 30 cc. of water and evaporated to a very thick syrup which crystallized on standing in a desiccator over phosphorus pentoxide and soda-lime. This residue, after being stirred with anhydrous ether, absolute alcohol, and petroleic ether, was filtered. It was finely powdered and digested and washed with ether. Yield 1.4 gm. M.p. = 165–166° with decomposition.

No. 143.

$C_{10}H_{14}O_2NCl$ (215.63).

Calculated. N 6.50, Cl 16.45.

Found. " 6.16 (Kjeldahl), Cl 16.70 (Carius).

The compound was not further purified.

3,6-Dimethyl-2,5-Di-(p-Hydroxybenzyl)-Pyrazine.—1.08 gm. (0.005 mol) of aminoketone hydrochloride were dissolved in 5 cc. of water. On addition of 0.35 cc. of ammonium hydroxide (*d* 0.90), a solid precipitate was obtained, which was stirred and washed with water and dried over phosphorus pentoxide. Yield 0.7 gm. It was crystallized from a water-pyridine mixture.

No. 153.

$C_{20}H_{26}N_2O_2$ (320.28). Calculated. C 74.96, H 6.30, N 8.75.

Found. " 74.98, " 6.41, " 8.84 (Dumas).

Acetylphenylmethylaminoacetic Acid.—16.1 gm. of phenylmethylaminoacetic acid gave 19.6 gm. of crude crystalline product. It was recrystallized from 30 gm. of acetone. The crystals which deposited were washed with anhydrous ether. They were united with the successive fractions obtained by concentrating the mother liquors. The combined product melted at 190–193°.

2 gm. were recrystallized from 7 gm. of boiling absolute alcohol in the presence of some norit. The crystals were washed with ether. M.p. = 192–193.5°.

No. 148.

$C_{11}H_{13}O_3N$ (207.17).

Calculated. C 63.75, H 6.32, N 6.76.

Found. " 64.02, " 6.45, " 6.55 (Kjeldahl).
" 0 (Van Slyke).

Phenylmethylaminoacetic Acid.—The preceding compound yielded this amino acid on hydrolysis. 2.1 gm. of acetylphenylmethylaminoacetic acid were refluxed for 1 hour with 30 cc. of hydrochloric acid. The solution was diluted with 30 cc. of water and evaporated under reduced pressure to complete dryness. The solution of this residue in 15 cc. of water was decolorized with norit and made slightly alkaline with ammonium hydroxide. On addition of alcohol the amino acid crystallized. It was washed with water, alcohol, then with ether, and dried at 100°.

No. 151.

$C_9H_{11}O_2N$ (165.15). Calculated. C 65.42, H 6.72, N 8.48.

Found. " 65.73, " 6.45, " 8.33 (Kjeldahl).

SUMMARY.

1. The action of acetic anhydride and pyridine on phenylaminoacetic acid, phenylalanine, and *l*-tyrosine has been studied. The main products of the reaction are carbon dioxide and derivatives of acetylaminacetone of the formula $R-CH(NH \cdot COCH_3) \cdot COCH_3$. With phenylmethylaminoacetic acid no loss of carbon dioxide occurs; simple acetylation takes place.

2. The constitution of the ketonic compounds has been established by their conversion into the corresponding hydrochlorides and pyrazines.

3. A reaction mechanism is suggested to explain the observed facts.

COMPOSITION OF BONE.

I. ANALYTICAL MICRO METHODS.*

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There are no methods in the literature for the quantitative estimation of the inorganic constituents of very small amounts of calcified material. Kramer and Howland (1) recently described improved methods of bone analysis, but their procedure calls for the use of 250 to 500 mg. of bone powder for each determination of carbonate.

With the technique described here a single determination of carbonate is performed on about 20 mg. of bone powder; 50 mg. suffice for the determination in duplicate of carbonate, calcium, and inorganic phosphorus. These three determinations, in duplicate, have been performed in some instances on half this amount of bone powder. This has enabled us to analyze minute specimens, such as the head bones of a human fetus which when dried and pulverized weighed 17.7 mg., and the calcified cusp of an aortic valve which gave 21.3 mg. of bone powder.

Preparation of Material.

The bones (or other calcified tissues) were removed from the body immediately after death. The adherent soft tissues were dissected from the bones which were then crushed into small fragments. The crushed bones were immediately extracted in a Soxhlet extraction apparatus with a mixture of ethyl alcohol and ether for about 4 hours. Before beginning the extraction this mixture was made alkaline with NaOH. Destruction of bone

* A preliminary report was presented at the Twenty-First Annual Meeting of the American Society of Biological Chemists, April, 1927 (*J. Biol. Chem.*, 1927, lxxiv, p. ix).

carbonate during the extraction because of contact with volatile acids was thus avoided since only the condensed vapors from the alkaline mixture came into contact with the bones.

The alcohol and ether were removed from the extracted bones by evaporation at a temperature not exceeding 38°. The bones were then pulverized finely and passed through an 80 mesh sieve. The preparation of the material for analysis was completed within 24 hours after removal of the specimen from the body.

Determination of Carbonate.

Carbonate was determined in Van Slyke's (2) manometric blood gas apparatus. Some difficulty was experienced in the introduction of the sample, for the particles of bone powder tended to adhere to the shoulder at the bottom of the cup (see (2), Fig. 4). The construction of the cup and of the stop-cock just below the cup was therefore modified; these modifications facilitated the complete introduction of the bone powder. The shoulder and capillary at the bottom of the cup were eliminated, and instead of a stop-cock with a 1 mm. bore, we employed one in which the bore tapered from 4 mm. at the upper end to 2 mm. at the lower end. The rest of the apparatus was of standard design.

For analysis sufficient bone powder was taken to liberate about 100 mm. of CO₂ as read on the closed manometer scale. In general, about 20 mg. sufficed. The sample was weighed on a balance weighing to 0.05 mg., a small piece of lead foil (4 cm. × 3 cm.) being used instead of weighing paper. Weighing paper was found unsatisfactory for this purpose, since it was observed that its weight occasionally fluctuated. The cause of these fluctuations was not determined; they may have been due to changes in moisture content or, more probably, to electrostatic forces.

About 0.5 cc. of mercury was introduced at the bottom of the cup and 2 drops of distilled water were placed on top of the mercury. The bone powder was then poured into the cup. No attempt was made to remove the powder completely from the lead foil, since the weight of the sample was obtained by difference after weighing the empty lead foil.

When properly introduced, most of the powder fell on the water at the base of the cup; very little or none adhered to the upper wall. By suitable manipulation, the powder was drawn

into the 50 cc. chamber. Care was taken to keep the powder in the upper half of this chamber. 2 or 3 cc. of mercury were run into the cup; then 2 drops of water were placed on top of the mercury and the level of this column was slowly lowered until the cup was empty. This method of washing reduced greatly the amount of water necessary for washing in the sample since the column of mercury with a thin film of water on top was equivalent to an equal column of water. The washing was continued until no particles of powder were visible on the sides of the cup. About 1 cc. of water generally sufficed when it was used a drop or two at a time in this way.

After the sample was washed in, the level of the water was raised almost to the upper stop-cock to expel most of the air from the chamber. It was considered unnecessary to remove all the air above the solution since the CO_2 evolved was determined by absorption with alkali. The dissolved gases in the 1 cc. of water were not removed; this was taken care of by means of a blank analysis. 7 cc. of approximately 3.5 N H_2SO_4 were poured into the cup, and 5 cc. admitted to the chamber, the remaining 2 cc. of acid serving as a seal. Mercury was then run into the cup, displacing the 2 cc. of acid. Enough of this mercury was admitted to fill the bore of the stop-cock and seal the chamber. The acid remaining in the cup was then removed. After the addition of the acid, the mercury in the chamber was lowered almost to the 50 cc. mark. The acid was then allowed to remain in contact with the powder for about 30 minutes or until no more bubbles of CO_2 were liberated. The contents of the chamber were then shaken for about 5 minutes and the pressure read at the 2 cc. mark. The level was again lowered, shaking was continued for 2 minutes, and the pressure again read. This was repeated until all the CO_2 had been liberated as evidenced by no further increase in pressure. Usually after the second or third shaking a constant pressure (p_1) was obtained. 5 cc. of approximately 13 N KOH were now poured into the cup. 2 cc. were admitted to absorb the CO_2 , and the upper stop-cock sealed with mercury as described above. As a result of the reaction between the strong acid and base, considerable heat developed. The solution was, therefore, shaken for 2 minutes to obtain a uniform temperature. The pressure was then read again (p_2).

Calculations.—It is possible to calculate the CO_2 content of the

bone directly from the gas laws. The volume of the gas is 2 cc., the pressure is the difference between p_1 and p_2 , and the temperature is taken as the mean of the two temperatures before and after absorption. Since the difference in the two temperatures is about 1° , no great error is thus introduced. From the volume of evolved CO_2 , reduced to 0° and 760 mm., and from the weight of the sample, the per cent of CO_2 is readily calculated.

It is, however, more convenient to utilize the tables prepared by Van Slyke and Sendroy (3). When the per cent of CO_2 is calculated from the following formula the same result is obtained as is given by direct calculation from the gas laws.

$$\frac{\text{Mm. pressure} \times \text{factor} \times 4.4}{\text{Weight of sample in mg.}} = \text{per cent of } \text{CO}_2$$

The pressure = $p_1 - p_2 - c$ where p_1 and p_2 are the pressures in mm. before and after absorption and c is the blank. The blank used was the value obtained by performing the operations described above without the use of bone powder. The difference in pressure before and after absorption in the blank analyses is the correction c ; this amounted to about 3 mm.

The factor employed is that taken from Van Slyke and Sendroy (3) (the last column of Table X, p. 142). In selecting the factor, the average temperature is employed as stated above. It can readily be shown that the above convenient expression is theoretically valid, and that it gives the same values as direct calculation.

Determination of Calcium and Phosphorus.

Again determined by difference, about 10 mg. of bone powder were weighed and transferred to a dry 10 cc. volumetric flask. 2 cc. of 1 M HCl were added and the mixture was then digested in a boiling water bath for about 10 minutes. When cool, 3 cc. of 20 per cent trichloroacetic acid were added and the volume was made up to 10 cc. with water. The solution was then filtered through a small funnel with a diameter at the top of about 2.5 cm. Munktell's filter paper No. 00 was used. To insure the absence of acid-extractable material, the filter paper was washed freely, first with HCl and then with water. When dry it was cut down to the desired size.

Calcium was determined in duplicate on 2 cc. aliquots of this filtrate according to the method of Kramer and Tisdall. The pH of the solution was adjusted with brom-cresol purple after addition of the ammonium oxalate.

The filtrate from the bone solutions was often turbid. A number of methods for removing the turbidity were tried without success. The following procedure was finally found to give a clear filtrate in every instance.

A 2 cc. aliquot of the first filtrate was transferred to a 10 cc. volumetric flask, and 5 cc. of molybdic acid reagent were added. This reagent is the one employed in the Briggs-Bell-Doisy method for phosphorus. The volume was made up to the mark with water. After standing for about half an hour, it was filtered in the same fashion as the first solution. For the phosphorus determination, 4 cc. aliquots of this second filtrate were used. To each of two 4 cc. aliquots in a 10 cc. volumetric flask were added 1 cc. of sulfite and 1 cc. of hydroquinone according to the Briggs-Bell-Doisy method. The volume was made up to 10 cc. and the color compared with the standard.

At the beginning of the investigation, if the first filtrate was clear, phosphorus was determined directly on 2 cc. aliquots. It frequently happened that some clear filtrates subsequently developed a turbidity during the development of the blue color. This turbidity seriously interfered with the accuracy of the results. The addition of the molybdic acid reagent and the second filtration were therefore adopted as a routine procedure whether the first filtrate was clear or not.

In making up the standard comparison solutions the same amounts of trichloroacetic acid and hydrochloric acid were added to the known solution as were contained in the aliquots of the unknown. In this way errors due to differences in pH and to the effect of organic substances in the reagents as pointed out by Stanford and Wheatley (4) were ruled out. The standard solution employed contained 0.025 mg. of P per cc. Usually 2 cc. were taken for comparison. When the phosphorus content in a particular specimen is high, more of the standard solution may be used or smaller aliquots of the bone solution may be taken. In order to obtain accurate results with this technique the amount of phosphorus in the unknown and in the standard should not differ by more than about 30 per cent.

TABLE I.
Reproducibility of Acid Digestion.

Bone.	Sample.	Ca	P	<u>Residual Ca</u> <u>P</u>
		<i>per cent</i>	<i>per cent</i>	
A	a	24.6	10.6	1.98
		25.2	10.7	
		24.9	10.7	
	b	23.9	10.2	2.00
		24.2	10.1	
		24.1	10.2	
	c	24.6	10.6	1.99
		24.9	10.5	
		24.8	10.6	
B	a	13.7	6.0	2.05
		13.2	6.0	
		13.5	6.0	
	b	13.4	6.4	1.92
		13.2	6.2	
		13.3	6.3	
C	a	12.4	5.7	2.02
		12.8	5.6	
		12.6	5.7	
	b	13.2	6.1	2.00
		13.0	5.9	
		13.1	6.0	
D	a	19.7	8.8	2.02
		20.1	8.8	
		19.9	8.8	
	b	19.2	8.5	2.00
		19.4	8.7	
		19.3	8.6	
E	a	24.0	10.1	2.04
		24.5	10.2	
		24.3	10.2	
	b	24.6	10.2	2.03
		23.3	10.0	
		24.0	10.1	

Reproducibility of Acid Digestion.

Table I gives the reproducibility obtained by the acid digestion described above. Three 10 mg. samples of Bone A were digested separately in an identical manner and then analyzed, the calcium

TABLE II.
Effect of Molybdate Precipitation on Phosphorus Content.

Bone.	Filtrate.	P	$\frac{\text{Residual Ca.}}{\text{P}}$
		<i>per cent</i>	
I	1	14.3	1.82
		14.3	
		<u>14.3</u>	
	2	13.2	1.98
		13.2	
		<u>13.2</u>	
II	1	7.0	1.86
		6.8	
		<u>6.9</u>	
	2	6.5	1.94
		6.7	
		<u>6.6</u>	
III	1	11.7	1.76
		11.9	
		<u>11.8</u>	
	2	10.6	1.98
		10.4	
		<u>10.5</u>	
IV	1	11.8	1.80
		11.6	
		<u>11.7</u>	
	2	10.3	2.04
		10.5	
		<u>10.4</u>	

Ca:P in $\text{Ca}_3(\text{PO}_4)_2 = 1.94$.

and phosphorus in each sample being determined in duplicate. The agreement among three samples digested separately is about the same as that between aliquots of the same solution. Similar results were obtained with Bones B, C, D, and E.

The question arose as to whether the precipitate caused by the addition of the molybdic acid reagent to aliquots of the first filtrate adsorbed some of the inorganic phosphate. If this were the case, then erroneous values for the ratio residual Ca:P would be obtained. To see whether this occurred several normal bones were analyzed for calcium and CO₂ in duplicate as described above

TABLE III.
Comparison of Cold Digestion and Hot Digestion.

Bone.	Digestion.	Ca	P	$\frac{\text{Residual Ca}}{\text{P}}$
		<i>per cent</i>	<i>per cent</i>	
A	Hot.	23.9	10.2	2.00
		24.2	10.1	
		24.1	10.2	
	Cold.	24.1	10.3	1.97
		23.9	10.2	
		24.0	10.3	
E	Hot.	24.0	10.1	2.04
		24.5	10.2	
		24.3	10.2	
	Cold.	23.5	10.1	1.96
		23.4	10.2	
		23.5	10.2	
F	Hot.	20.1	9.1	2.01
		20.4	8.6	
		20.3	8.7	
			8.8	2.06
		20.2	8.6	
		20.7	8.7	
	Cold.	20.5	8.7	2.04
		20.8	8.9	
		20.8	8.9	
		20.8	8.9	

while phosphorus was determined on aliquots of both the first and second filtrates in each case. The final blue phosphate solutions obtained from the second filtrates were always clear, while those from the first filtrates were more or less turbid. The former gave excellent color matches with the standard, while the latter were more or less off color. The results are given in Table II. Ratios

calculated from the second filtrate are 1.98, 1.94, 1.98, and 2.04 while from the first filtrate they are 1.82, 1.86, 1.76, and 1.80. In normal bone the ratio is about 1.94 according to Howland, Marriott, and Kramer (5). These results show that the second precipitation and filtration do not remove sufficient inorganic phosphorus to give erroneous values for the ratio.

When the molybdate reagent is added to an aliquot of the first filtrate, the precipitate that forms on standing is flocculent and appears large in amount. It was found that cold acid digestion greatly diminished the amount of organic material that went into solution. When the bone solutions were prepared according to the technique described previously except that the digestion with acid was carried out by shaking in the cold, very little organic material dissolved. This was shown by the very small amount of precipitate subsequently obtained when the molybdate reagent was added. The same analytical results were obtained with both hot and cold acid digestions as is shown in Table III.

However, when cold digestion was used the amount of precipitate produced by molybdate was so small in some cases that it did not coagulate, and on filtration, passed into the filtrate. This turbidity in the second filtrate interfered with the phosphate analysis. Hot digestion was therefore used as a routine, as it invariably resulted in a clear second filtrate.

Effect of Delaying Ether-Alcohol Extraction.

The length of time elapsing between removal of the specimen from the body and the completion of the preparation for its analysis is another possible source of error. This of course is a matter not peculiar to the micro technique, but to any method of bone analysis. If the bones, after removal from the body, are permitted to stand for some time before the extraction with ether and alcohol, it is possible that some of the carbonate may be decomposed, and some of the CO_2 lost. There is also the possibility to be considered that the percentage of inorganic phosphate may undergo some change.

Accordingly, a quantity of normal bone, Bone A, was crushed and divided into two portions immediately after removal from the body. One portion was extracted, dried, and pulverized on the day of autopsy. The other portion was put into a cork-stoppered

bottle and was set aside for 5 months. It remained in this bottle at room temperature throughout the warmest part of the year, from April to September. After 5 months it was extracted and prepared for analysis in the usual manner.

The results of the analysis are given in Table IV. It is seen that the content of calcium, inorganic phosphorus, and CO₂ had not altered materially as a result of this long delay. Bone E is another specimen of normal bone which was tested in the same way with similar results. In these two instances standing unextracted for months did not materially affect the composition.

TABLE IV.
Effect of Delaying Ether-Alcohol Extraction.

Bone.	Time extracted.	Ca	P	CO ₂	Residual Ca P
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	
A	Immediately.	24.6	10.6	4.05	1.98
		25.2	10.7	4.16	
		24.9	10.7	4.11	
	After 5 mos.	24.9	10.3	4.07	2.04
		24.8	10.5	3.99	
E	Immediately.	24.9	10.4	4.03	2.04
		24.0	10.1	3.79	
		24.5	10.2	3.86	
	After 5 mos.	24.3	10.2	3.83	1.98
		24.1	10.6	3.90	
		24.7	10.4	3.94	
		24.4	10.5	3.92	

This was contrary to our expectations. It may well be, however, that other kinds of bones, or even the same bones, under somewhat different conditions may undergo considerable change if they are allowed to stand unextracted.¹ To avoid this possibility, we

¹ In one instance we did obtain abnormal results. Fragments of rat bone were allowed to stand in an open dish for several months. Analysis of this material gave

Ca <i>per cent</i>	P <i>per cent</i>	CO ₂ <i>per cent</i>	$\frac{\text{Residual Ca}}{\text{P}} = 2.35$
10.7	4.0	1.79	
11.0	4.0	1.42	
10.9	4.0	1.61	

adopted the policy of extracting the calcified specimen immediately after removing it from the body.

Reproducibility of CO₂ Analyses.

Table V gives the results for CO₂ on various calcified tissues containing different amounts of CO₂. The greater the CO₂ content the smaller of course will be the sample required to liberate 100 mm. of CO₂ in the closed manometric apparatus. Samples much smaller than 5 mg. should not be used as then an error of 0.1 mg. in weighing will give more than a 2 per cent error in the weight of the sample. The reproducibility of the CO₂

TABLE V.
CO₂ Analysis of Bones.

Specimen.	Weight of sample.	CO ₂	Specimen.	Weight of sample.	CO ₂
	<i>mg.</i>	<i>per cent</i>		<i>mg.</i>	<i>per cent</i>
G	21.6	1.83	J	10.8	4.66
	21.7	1.88		8.9	4.70
H	21.7	2.33	K	18.8	3.02
	23.0	2.13		6.5	3.03
				6.6	2.95
I	10.5	3.63	L	6.2	2.60
	11.2	3.69		4.3	2.56

determination was as good as those of the calcium and phosphorus determinations.

Ratio, Residual Ca:P.

The ratio, residual Ca:P, was calculated in a manner similar to that of Howland, Marriott, and Kramer (5). The per cent of CO₂ multiplied by 0.91 gave the per cent of calcium assumed to be present as carbonate calcium. The residual calcium was obtained by subtracting the carbonate calcium from the total calcium, all expressed in per cent or gm. per 100 gm. of bone. Thus

$$\frac{\text{Residual Ca}}{\text{P}} = \frac{(\text{total Ca}) - (\text{carbonate Ca})}{\text{total inorganic P}}$$

Table VI is a summary of the ratios obtained in forty-four analyses performed with this micro technique. The mean obtained was 2.00 ± 0.01 . The value for *a.d.*, the average deviation, was obtained in the usual way; *A.D.* has its usual signifi-

TABLE VI.
Summary of Ratios.

$\frac{\text{Residual Ca}}{\text{P}}$	Deviation.	$\frac{\text{Residual Ca}}{\text{P}}$	Deviation.
1.98	-0.02	1.96	-0.04
1.99	-0.01	1.97	-0.03
2.00	0.00	1.97	-0.03
1.97	-0.03	1.90	-0.10
2.04	+0.04	1.88	-0.12
2.04	+0.04	2.12	+0.12
2.03	+0.03	2.00	0.00
1.96	-0.04	2.02	+0.02
1.98	-0.02	2.21*	+0.21
1.91	-0.09	2.02	+0.02
1.91	-0.09	2.00	0.00
2.01	+0.01	2.08	+0.08
1.86	-0.14	1.93	-0.07
1.95	-0.05	2.12	+0.12
1.96	-0.04	2.12	+0.12
1.98	-0.02	1.94	-0.06
1.89	-0.11	2.05	+0.05
1.95	-0.05	1.92	-0.08
2.05	+0.05	2.10	+0.10
1.82	-0.18	2.00	0.00
		2.14	+0.14
		2.10	+0.10
		2.03	+0.03
		2.01	+0.01
		Mean = 2.00	<i>a.d.</i> = ± 0.06 <i>A.D.</i> = ± 0.01

Residual Ca:P = 2.00 ± 0.01 . Ca:P in $\text{Ca}_3(\text{PO}_4)_2$ = 1.94.

* Two other analyses of the same bone gave ratios of 2.02 and 2.00.

cance, *i.e.* $A.D. = \frac{a.d.}{\sqrt{n}}$. An obviously erroneous value of 2.21

was included for the sake of completeness although two other analyses, each in duplicate, of the same specimen of bone powder

gave ratios of 2.02 and 2.00. This table contains all the ratios which we have obtained on normal rat bone; none has been omitted. It also contains all the ratios which we have obtained on pathologically calcified human tissues with the exception of the three ratios given in Table II of the following paper (6). The ratios in this table exhibit the normal distribution of deviations about a mean; very high values and very low values are rare. The mean value of 2.00 ± 0.01 does not differ widely from 1.94, the Ca:P value usually given for normal bone; it is about 3 per cent higher than the theoretical value of 1.94 for $\text{Ca}_3(\text{PO}_4)_2$.

DISCUSSION.

The numerator of our ratio is the same as that of Howland, Marriott, and Kramer; *i.e.*, residual Ca is the per cent of calcium remaining after the carbonate calcium is deducted from the total calcium. For the denominator we have used the total amount of inorganic phosphorus. This is different from the calculation of Howland, Marriott, and Kramer, who use as the denominator the residual phosphorus obtained by deducting from the total phosphorus the amount calculated as bound to magnesium in the compound $\text{Mg}_3(\text{PO}_4)_2$.

These authors showed ((5), p. 724) that for a typical bone, the per cent of magnesium present was 0.24. If this is all present as $\text{Mg}_3(\text{PO}_4)_2$, then the per cent of phosphorus in this bone present as magnesium phosphate is 0.3. For an error of ± 3 per cent in the phosphorus determination, they found for this bone that the total phosphorus in per cent was 11.6 ± 0.35 . Thus the amount of phosphorus bound as magnesium phosphate is about the same as the error in the determination of total inorganic phosphorus.

This probably accounts for the fact that we obtained normal ratios even though the magnesium analysis was omitted. With very small specimens, which do not contain enough material for a complete analysis, omission of the magnesium determination is therefore preferable to omission of any of the other three.

For the CO_2 analysis enough sample was taken to obtain about 100 mm. of CO_2 . As stated previously, about 20 mg. of bone powder were usually sufficient. When only about 20 mg. were available for the determination of CO_2 we preferred to make the analysis in duplicate using half of the available quantity for each

analysis rather than rely on a single analysis. Of course, with half the stated quantity of CO_2 , the relative error is doubled since the absolute errors remain the same. The Ca:P ratio, however, is not markedly affected even when the error in CO_2 is doubled, for the error in residual Ca is increased by only about one-fifth of the increase in the error in CO_2 .²

The occurrence of turbidity immediately after addition of molybdic acid or during the development of the color in the colorimetric determination of phosphorus has been observed by Bell and Doisy (7), Briggs (8), Benedict and Theis (9), and Fiske and Subbarow (10). The turbidity was ascribed to the precipitation of some unknown substance or substances present in urine and blood. Briggs suggested that the precipitate may be due to the interaction between molybdic acid and a small amount of protein which escaped precipitation because of insufficient shaking after the trichloroacetic acid was added. To avoid the turbidity he recommended vigorous shaking and a delay of 10 minutes before the precipitated proteins were filtered off. Fiske and Subbarow were of the opinion that a small amount of protein escaped precipitation as a result of too low a concentration of trichloroacetic acid, and was subsequently precipitated by phosphomolybdic acid. They recommended an increase in the concentration of trichloroacetic acid.

The above observations were made on filtrates from blood and urine. As far as bone solutions are concerned, the nature of the precipitate which forms on addition of molybdic acid is unknown. The amount of precipitate formed on addition of trichloroacetic

² In adult rats $\frac{\text{carbonate Ca}}{\text{total Ca}} = 15$ per cent approximately, therefore, when total Ca = 28.00 per cent then carbonate Ca = 4.00 per cent and residual Ca = 24.00 per cent. When the error in the CO_2 analysis is

	2 per cent per cent	4 per cent per cent
Total Ca	= 28.00	= 28.00
Carbonate Ca	= 4.08	= 4.16
Residual Ca	= 23.92	= 23.84

Thus an error of 2 per cent in the CO_2 causes an error in residual Ca of 1 part in 240 or about 0.4 per cent; and a 4 per cent error in CO_2 gives an error of 2 parts in 240 or about 0.8 per cent in residual Ca, i.e. only about one-fifth the error in CO_2 .

acid to the original bone solution is small; it is very much less in amount than that obtained in similar procedures with blood. Upon addition of molybdic acid to the first filtrate, variable amounts of turbidity and precipitate are obtained from the same bone powder, depending upon the temperature at which the bone was digested with HCl. This points to the presence in the filtrate of intermediate products of protein decomposition. In this connection it is of interest to note that Hiller and Van Slyke (11) found that while trichloroacetic acid precipitated proteins, it permitted intermediate products such as "proteoses" and "peptones" to pass into the filtrate.

In any case the same phosphorus values and normal Ca:P ratios were obtained whether the molybdate precipitate was comparatively large in amount (as in the hot digestions) or very much smaller in amount (as in the cold digestions). This can be seen from Table III. Thus the amount of phosphate removed by this precipitate, whether by chemical combination or by adsorption, appears either to be negligible or to be within the limit of error of the method used to determine phosphorus.

The mean value of 2.00 ± 0.01 is somewhat higher than the ratios obtained by Howland, Marriott, and Kramer. It is near enough, however, to their value to be regarded as satisfactory for a micro technique.

While these procedures have been developed to permit analysis of very small specimens of calcified tissues, they may also be used for determining CO₂, calcium, and phosphorus in solids other than bone.

SUMMARY.

1. A technique is described for determining the CO₂ content of bone for which only about 20 mg. of bone powder are required.

2. A technique is described for determining in duplicate both calcium and inorganic phosphorus in bone in which only about 10 mg. of bone powder are required.

3. With the procedures outlined here, CO₂, calcium, and inorganic phosphorus in bone may be determined in duplicate on about 50 mg. of material; the entire procedure has been performed on as little as 17.7 mg.

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COMPOSITION OF BONE.

II. PATHOLOGICAL CALCIFICATION.*

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(Received for publication, June 2, 1928.)

By means of the micro technique described in the preceding communication (1) we have analyzed a number of minute specimens of pathological calcification obtained from humans. The analytical results are given in Tables I and II.

The last column in Table I shows that these specimens have normal ratios; i.e., the same Ca: P ratio, within the experimental deviation, as is found in normal bones.

One type of pathological calcification, however, seems to have a composition different from normal bone. Table II gives the analytical results for three specimens of calcified fibroid of the uterus obtained from three different individuals. The ratios 2.23, 2.23, and 2.18 do not appear to be attributable to experimental error. In the case of fibroid Specimen A, there were two kinds of calcification in the specimen: one a chalky deposit, "chalk," and the other a tough, firm deposit, "hard." These were analyzed separately. The former gave a normal value of 1.98, while the latter gave an abnormal value of 2.23.

In his Harvey Lecture for 1911 Wells (2) stated that the composition of pathological calcific deposits is the same as that of normal bone. Recently Wells (3) again stated: "The composition of the inorganic salts in calcified areas in the body seems to be practically the same, if not identical, whether the salts are laid down under normal conditions (ossification) or under patho-

*Presented before the Society for Experimental Biology and Medicine, New York, November, 1927 (*Proc. Soc. Exp. Biol. and Med.*, 1927, xxv, 141).

logical conditions." The only instance in which it was reported that the ratio of Ca: P was different from that of normal bone was that given by Eden (4). Eden found that in young callus the ratio Ca: P was greater than in mature bone, and that as the callus became older and was replaced by true bone the ratio

TABLE I.
Pathological Calcification (Human).

Specimen.	Ca	P	CO ₂	$\frac{\text{Carbonate Ca}}{\text{Total Ca}}$	$\frac{\text{Residual Ca}}{\text{P}}$
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	
Thyroid.	20.1	9.1	3.01		
	20.2	9.1	3.18	14	1.91
	20.2	9.1	3.10		
Tuberculous lymph nodes.	31.7	15.0	4.66		
	32.9	14.7	4.70	13	1.88
	32.3	14.9	4.68		
Cusp of aortic valve.	27.4	11.6	4.48		
	27.4	11.6	4.50	15	2.01
	27.4	11.6	4.49		
Capsule of spleen.	23.8	10.4	4.63		
	23.3	10.5	4.41	17	1.86
	23.6	10.5	4.52		
Lung lymph node.	33.1	14.8	4.76		
	33.2	14.7	4.89	13	1.95
	33.2	14.8	4.83		
Mesenteric lymph node.	31.1	13.6	4.70		
	30.6	13.6	4.79	14	1.96
	30.9	13.6	4.75		
Fibroid Specimen A (chalk).	31.7	13.2	5.84		
	31.3	13.2	5.70	17	1.98
	31.5	13.2	5.77		

approached that of mature bone. His results have been confirmed by Herrmann (5).

These results, however, are not comparable with ours because of the different analytical procedures employed (6). Their values for P are those for total phosphorus and not for inorganic phos-

phorus, and their values of Ca are those for total calcium and not for residual calcium. The latter quantity cannot be calculated from their data since they reported no CO₂ analyses.

The abnormally high ratios of 2.23, 2.23, and 2.18 obtained in the case of calcified fibroids of the uterus take on added interest when compared with the high ratio of 2.23 ± 0.03 obtained for primary calcification. The possible significance of these high ratios is discussed in a subsequent paper (7).

TABLE II.
Calcified Fibroid of Uterus (Human).

Specimen.	Ca	P	CO ₂	$\frac{\text{Carbonate Ca}}{\text{Total Ca}}$	$\frac{\text{Residual Ca}}{P}$
	per cent	per cent	per cent	per cent	
A (hard).	23.6	8.9	3.72		
	23.8	9.2	3.69	14	2.23
	23.7	9.1	3.71		
B	29.9	11.6	4.09		
	29.7	11.7	4.11	13	2.23
	29.8	11.7	4.10		
C	26.1	10.2	4.22		
	25.7	10.2	3.96	14	2.18
	25.9	10.2	4.09		

SUMMARY.

1. Seven specimens of pathologically calcified tissues were analyzed with the micro technique; all gave normal values for the ratio residual Ca : P.

2. Abnormal values for this ratio were obtained in analyses of calcified fibroids of the uterus.

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COMPOSITION OF BONE.

III. PHYSICOCHEMICAL MECHANISM.*

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INTRODUCTION.

A number of theories have been advanced to account for the deposition of bone salts. Freudenberg and György (1) consider that calcium first deposits as an organic calcium compound which then unites with phosphate, and that subsequently the calcium-organo-phosphate decomposes to give calcium phosphate. Dhar (2) attempts to explain it mainly from the point of view of colloidal phenomena. Robison (3) and his coworkers assign a prominent rôle in calcification to an enzyme in bone that converts organic phosphorus compounds into inorganic phosphates. Wells (4) and others have suggested that a local decrease in the CO_2 content or in the acidity of the tissue fluids may account for calcification.

The deposition of bone salts has also been attributed to simple precipitation of insoluble salts. Holt, La Mer, and Chown (5) in their notable investigation were the first to apply the modern theories of solution to this problem. More recently, Hastings, Murray, and Sendroy (6) studied the solubilities of calcium carbonate and tertiary calcium phosphate. These latter studies were also made from the point of view of the theory of complete dissociation of electrolytes, and constitute an important advance in that they contributed improved theoretical considerations.

From these last two investigations it appears evident that the ion product of $\text{Ca}_3(\text{PO}_4)_2$ is not the quantity which governs the deposition of calcium phosphate, and that the mechanism of calcification still remains obscure. This might be taken to mean that solubility product considerations are not applicable to this problem and that the solution must be sought elsewhere. However there is another alternative. It is possible that valuable

* Presented before the Society for Experimental Biology and Medicine, New York, January, 1928 (*Proc. Soc. Exp. Biol. and Med.*, 1928, xxv, 283).

information regarding calcification may yet be obtained with the aid of ion products if we employ the ion product of CaHPO_4 instead of that of $\text{Ca}_3(\text{PO}_4)_2$.

Knowledge of the composition of primary calcification, *i.e.* of the composition of the bone salts immediately after deposition, would probably throw considerable light on the mechanism involved in calcification. The attempts which have been made to elucidate the nature of the processes concerned in the deposition of bone salts have been made on the basis of analyses of old bone. There are a number of considerations which led us to suspect that the composition of primary calcification may be different from that of old bone. Just as precipitates of calcium phosphate change in composition when they remain in contact with the solutions from which they have been precipitated, so it seemed possible that the calcium salts after being deposited in the cartilage might subsequently undergo a change in composition to that obtained on analysis of old bone.

In the fourth paper of this series we give the results of analyses of primary calcification which were performed with our micro technique. In this paper we present the considerations which led us to suspect that $\text{Ca}_3(\text{PO}_4)_2$ is not deposited from serum and that new calcification may differ in composition from old bone. In outline there are: (1) the presence of $\text{Ca}_3(\text{PO}_4)_2$ in bone has never been demonstrated; (2) a chemical compound of the formula $\text{Ca}_3(\text{PO}_4)_2$ has never been prepared even from simple inorganic solutions; (3) the ion product $[\text{Ca}^{++}]^3 \times [\text{PO}_4^{=}]^2$ does not appear to be the quantity which determines calcification, either *in vivo* or *in vitro*; (4) when solutions of calcium and phosphate are mixed the initial precipitate is frequently CaHPO_4 ; this precipitate changes, on standing, to a solid phase whose empirical composition approximates $\text{Ca}_3(\text{PO}_4)_2$; (5) calcification is not obtained experimentally or clinically when the ion product $[\text{Ca}^{++}] \times [\text{HPO}_4^{=}]$ is markedly below $K'_{s.p.} \text{CaHPO}_4$;^{*} calcification is obtained when the ion product for CaHPO_4 is close to $K'_{s.p.} \text{CaHPO}_4$; (6) the experimental validity of the $\text{Ca} \times \text{P}$ product indicates that calcification is due to a second order reaction.

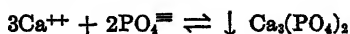
^{*} The roman and italic letters for K in the forms $K_{s.p.}$ and $pK_{s.p.}$, *etc.*, are used interchangeably in this paper; there is no special significance attached to the italic form.—Publisher's note.

Precipitation of Calcium Phosphate.

The precipitation of calcium phosphate from both inorganic and biological systems has been investigated from the point of view of the ion product $[Ca^{++}]^3 \times [PO_4^{==}]^2$ and $K'_{s.p.}$ $Ca_3(PO_4)_2$. The results obtained show that these quantities are either inapplicable or inadequate. Holt, La Mer, and Chown postulated supersaturation with respect to $Ca_3(PO_4)_2$ in order to account for the peculiarities observed. Sendroy and Hastings concluded that the theory of supersaturation is inadequate. Klinke (7), Robison and Soames (3), and Greenwald (8) have presented additional evidence against the theory of supersaturation. Moreover, the experiments of Shipley, Kramer, and Howland (9), of Kramer, Shelling, and Orent (10), and of Shipley and Holt (11) have demonstrated that aqueous solutions of the same inorganic composition as serum may hold in solution, without the aid of organic compounds, the same amounts of calcium and phosphate as are found in serum.

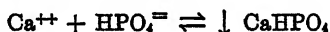
In discussing the composition of the bone salts it is generally taken for granted that the calcium phosphate is present as $Ca_3(PO_4)_2$. We wish to emphasize that *the presence of $Ca_3(PO_4)_2$ in bone has yet to be demonstrated*. An examination of the literature (Dolhaine (1), Robison and Soames (3), and (12-34)) shows that the nature of the calcium phosphate compound in bone has never been elucidated. The belief that bone contains $Ca_3(PO_4)_2$ has gained universal acceptance apparently only on the basis of the empirical Ca : P ratio obtained from analyses. Moreover, when the analytical data are examined, it is seen that it has never been demonstrated that bone contains an empirical Ca:P ratio which is the same as that of $Ca_3(PO_4)_2$.

There are astonishingly few analyses of bone which have been made on unashed material by accurate analytical procedures. These very scanty data, as well as the numerous analyses of ashed material, show a Ca : P ratio only *approximating* that of $Ca_3(PO_4)_2$. The deviations from the theoretical ratio have been ascribed to errors in experimental technique. This may or may not be so, but it is striking that a similar situation exists as regards tricalcium phosphate prepared by precipitation from solution. This reaction is usually written



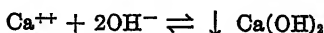
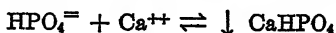
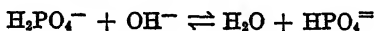
We have been unable to find any evidence of the existence of a molecular species with the formula $\text{Ca}_3(\text{PO}_4)_2$. Precipitates of this "substance" rarely have the correct empirical composition and they cannot be purified by recrystallization. The evidence is such as to lead us to suspect that there may be no such chemical entity as $\text{Ca}_3(\text{PO}_4)_2$. No one has succeeded in preparing it by precipitation (12, 35-48), bearing out the theoretical objections to such a reaction on the grounds that fifth order reactions do not occur (49). We do not take the position that there can be no compound with this formula. It may be found possible to synthesize it by other methods, but so far no one has succeeded in preparing it by precipitation.

As a result of the foregoing considerations we have sought for another explanation of the mechanism of calcification. Dicalcium phosphate is also a slightly soluble substance. Moreover it is a definite molecular entity with characteristic crystal forms. In addition, the formation of a precipitate of CaHPO_4 is due to a second order reaction; *i.e.*,



It is generally agreed that the precipitate which is deposited from an acid solution is crystalline CaHPO_4 . The confusion exists only as regards the nature of the substance deposited from neutral or alkaline solutions. It is also generally agreed that when the precipitated CaHPO_4 is allowed to stand in contact with the supernatant solution, a subsequent reaction occurs—the solution becomes more acid and the solid phase becomes more basic. The confusion here is concerning the composition of the solid phase at the final equilibrium.

The titration curves of phosphoric acid obtained by various investigators (43, 50-52) constitute additional strong evidence for the precipitation of CaHPO_4 . Since the pH remains constant at about 7 during the addition of the 2nd and 3rd equivalents of $\text{Ca}(\text{OH})_2$, it means that approximately half of the total phosphate is present as H_2PO_4^- . The reactions which occur throughout this range therefore appear to be



Precipitates whose Ca : P ratio is greater than 1.29 are said to be mixtures, in varying proportions, of CaHPO_4 and $\text{Ca}(\text{OH})_2$ (39, 40).

Solubility Product of CaHPO_4 .

There have been a number of investigations dealing with the solubility of dicalcium phosphate, but the earlier work cannot be used in evaluating $K'_{s.p.} \text{CaHPO}_4$ because the pH of the solutions was not determined. Behrendt (53), Kugelmass and Shohl (54), Holt, La Mer, and Chown (5), and Domontovitch and Sarubina (55) have made determinations of $K'_{s.p.} \text{CaHPO}_4$ taking the pH of the solutions into consideration.

We have recalculated $K'_{s.p.} \text{CaHPO}_4$ from the data of the last three investigations mentioned above, using the expression

$$K'_{s.p.} \text{CaHPO}_4 = [\text{Ca}^{++}] \times [\text{HPO}_4^-] \quad (1)$$

We have set $[\text{Ca}^{++}]$ equal to the total calcium concentration as obtained by analysis. The value of $[\text{HPO}_4^-]$ was obtained by means of the expression

$$[\text{HPO}_4^-] = \frac{[\text{P}]}{1 + 10^{(\text{pK}_2' - \text{pH})}} \quad (2)$$

in which [P] is the concentration of phosphate obtained by analysis, and pK_2' was calculated from the expression given by Sendroy and Hastings

$$\text{pK}_2' = 7.15 - 1.25 \sqrt{\mu} \quad (3)$$

Equation (2) was obtained by rearrangement of equation (15) of Sendroy and Hastings ((6), p. 785).

When the values of $K'_{s.p.} \text{CaHPO}_4$ obtained in this way are plotted against $\sqrt{\mu}$, we obtain

$$\text{pK}'_{s.p.} \text{CaHPO}_4 = 6.4 - 2.3 \sqrt{\mu} \text{ for } 38^\circ \quad (3,a)$$

This linear expression describes the data from zero ionic strength up to values of 0.38 for $\sqrt{\mu}$; i.e., up to approximately the ionic strength of serum. For the range between room temperature and body temperature, up to $\sqrt{\mu} = 0.38$,

$$\text{pK}'_{s.p.} \text{CaHPO}_4 = 6.5 - 2.6 \sqrt{\mu} \text{ for } 19\text{--}38^\circ$$

These values are probably correct to ± 0.1 . At zero ionic strength, therefore,

$$pK_{s.p.} \text{ CaHPO}_4 = 6.4 \pm 0.1 \text{ at } 38^\circ$$

i.e.,

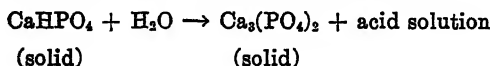
$$K_{s.p.} \text{ CaHPO}_4 = (4 \pm 1) \times 10^{-7}$$

The value $2.3 \sqrt{\mu}$ for $\Delta pK'_{s.p.}$ differs somewhat from the value $4 \sqrt{\mu}$ given by Holt, La Mer, and Chown. Our value for $K_{s.p.} \text{ CaHPO}_4$ at zero ionic strength agrees well with their value; we have, moreover, estimated the probable accuracy of this value.

Subsequent Reaction and Final Equilibrium.

When solutions containing calcium are mixed with solutions containing phosphate so that the resulting solution is acid, it is generally agreed that the precipitate which deposits from solution is secondary calcium phosphate. It is also generally agreed that this precipitate subsequently reacts with the supernatant solution; the solution becomes more acid and the solid phase more basic; *i.e.*, the ratio Ca: P in the solid phase becomes larger.

It has also been demonstrated by Rindell (56) and by Buch (57) that when crystals of secondary phosphate are equilibrated with water the composition of the solid phase changes. The nature and mechanism of these reactions are still unknown. The reaction has been represented, for example, as follows:



The formula $\text{Ca}_3(\text{PO}_4)_2$ appears frequently in such equations because the solid phase at equilibrium frequently has a Ca: P ratio approximating 1.94, the theoretical value for a compound with the formula $\text{Ca}_3(\text{PO}_4)_2$.

The conditions which determine the final equilibrium are unknown. Holt, La Mer, and Chown attempted to explain this behavior using $K'_{s.p.} \text{Ca}_3(\text{PO}_4)_2$ as the criterion of the final equilibrium, postulating prolonged supersaturation with respect to $\text{Ca}_3(\text{PO}_4)_2$. This theory, however, does not account satisfactorily for the experimental results.

TABLE I.
Solutions in Equilibrium with Precipitates at 38°.

Experiment No.*	$\sqrt{\mu}$	Ca <i>mols</i> $\times 10^3$	pH	Ca:P in solid phase.	$\log \frac{[Ca^{++}]}{[H^+]}$
†	0.025	0.20	6.40	2.06	2.7
CC 6	0.063	1.37	5.31	1.93	2.5
Z 1	0.064	1.46	5.30	1.94	2.5
Z 7	0.074	1.19	5.42	1.94	2.5
DD 1	0.075	1.65	5.15	1.94	2.4
CC 5	0.075	2.03	5.18	1.94	2.5
V 1	0.076	1.95	5.21	1.94	2.5
CC 4	0.084	2.74	4.96	1.96	2.4
Z 8	0.084	1.01	5.70	1.94	2.7
Z 2	0.085	1.48	5.46	1.94	2.6
†	0.085	2.50	5.06	1.94	2.5
DD 2	0.091	1.98	5.44	1.94	2.7
V 2	0.099	1.98	5.27	1.94	2.6
CC 3	0.101	3.44	5.00	1.91	2.5
M 9	0.103	3.50	4.87	1.94	2.4
DD 3	0.103	2.20	5.51	1.94	2.9
M 8	0.109	4.00	4.84	1.94	2.4
M 7	0.112	4.15	4.86	1.94	2.5
CC 2	0.112	4.15	5.04	1.94	2.7
M 6	0.120	4.55	4.94	1.94	2.6
CC 1	0.122	5.02	5.04	1.96	2.7
M 5	0.123	5.00	5.03	1.94	2.7
Z 3	0.127	1.56	5.55	1.94	2.7
S 2	0.13	5.70	5.08	1.26	2.8
M 4	0.13	5.30	5.06	1.29	2.8
V 3	0.163	1.99	5.33	1.94	2.6
Z 4	0.227	1.66	5.67	1.94	2.9
V 4	0.273	2.06	5.46	1.94	2.8
U 9	0.34	5.74	5.39	1.29	3.2
U 10	0.34	5.45	5.36	1.29	3.1
V 5	0.363	2.10	5.51	1.94	2.8
V 6	0.363	2.20	5.52	1.94	2.9
AA 11	0.374	0.115	7.70	2.05-2.20	3.8
AA 10	0.374	0.153	7.10	2.05-2.20	3.3
AA 9	0.376	0.125	7.40	2.05-2.20	3.5
AA 5	0.376	0.145	6.92	2.05-2.20	3.1
AA 8	0.377	0.095	7.29	2.05-2.20	3.3
AA 7	0.378	0.133	7.22	2.05-2.20	3.3
AA 6	0.380	0.153	7.22	2.05-2.20	3.4
AA 2	0.381	0.125	6.95	2.05-2.20	3.1

* The data are taken from Holt, La Mer, and Chown (5).

† The seventh experiment of Holt, La Mer, and Chown ((5), Table I, p. 515).

‡ The experiment performed 9 days after mixing ((5), Table I, p. 569).

TABLE I—*Concluded.*

Experiment No.*	$\sqrt{\mu}$	Ca <i>mols</i> $\times 10^3$	pH	Ca:P in solid phase.	$\log \frac{[Ca^{++}]}{[H^+]}$
AA 3	0.381	0.150	7.05	2.05–2.20	3.2
AA 4	0.381	0.123	7.05	2.05–2.20	3.1
AA 1	0.382	0.143	7.02	2.05–2.20	3.2
Z 5	0.445	2.00	5.94	1.94	3.2
T 7	0.509	3.07	5.53	1.94	3.0
Z 6	0.619	2.44	5.99	1.94	3.4
Y 4	0.65	8.31	6.28	1.29	4.2
Y 6	0.65	8.50	6.31	1.29	4.2

We have found an interesting regularity in all the experiments in which final equilibrium was attained by shaking precipitates of calcium phosphate with their supernatant solutions. When the analytically determined calcium concentration of the solution, expressed in mols per liter, is divided by the hydrogen ion concentration a constant is obtained. The results are shown in Table I. The last column shows that for a given ionic strength

$$\log \frac{[Ca^{++}]}{[H^+]^\dagger} = \text{a constant} \quad (4)$$

therefore

$$\frac{[Ca^{++}]}{[H^+]} = K'_{\text{equil.}} \quad (5)$$

Moreover $\log K'_{\text{equil.}}$ increases with increasing ionic strength in accordance with modern theories of solution. When the data are given graphically, it is seen that there is a straight line relationship between $\log K'_{\text{equil.}}$ and $\sqrt{\mu}$ in which, at 38° ,

$$\log K'_{\text{equil.}} = 2.4 + 2.2 \sqrt{\mu} \quad (6)$$

i.e.,

$$\log K_{\text{equil.}} = 2.4 \text{ at zero ionic strength} \quad (7)$$

† We have followed the notation of Michaelis with regard to hydrogen ion, in which "the symbol $[H^+]$ will represent not the concentration but the activity of H-ions, and pH its negative logarithm." (L. Michaelis, Hydrogen ion concentration, translated by W. A. Perlzweig, Baltimore, 1926, i, p. 128.) Hastings, Murray, and Sendroy used the activity symbols α_{H^+} and $p\alpha_{H^+}$.

or

$$K_{\text{equil.}} = 10^{3.4} \quad (8)$$

Furthermore this same ratio gives a similar constant at room temperature as can be seen from Table II. The ionic strength cannot be calculated for these solutions because the concentrations of the ions other than calcium, hydrogen, and phosphate are not given. The authors state however, that the "Gesamtkonzentration der Elektrolyte" ranged between 0.04 and 0.10 M.

TABLE II.

Solutions in Equilibrium with Precipitates at 19-22°.

Data of Domontovitsch and Sarubina ((55) p. 466).

Experiment No.	Ca	pH	$\log \frac{[\text{Ca}^{++}]}{[\text{H}^+]}$
	<i>mols</i> $\times 10^3$		
4	3.32	5.40	2.9
	1.30	5.86	3.0
	0.64	6.26	3.1
5	0.62	5.96	2.8
	1.60	5.52*	2.7
	1.40	5.54	2.7
	32.4	4.52	3.0
6	27.6	4.52	3.0
	25.7	4.64	3.1
	31.6	4.44	2.9
	26.2	4.48	2.9
	19.5	4.78	3.1
	30.0	4.42	2.9
	15.2	4.82	3.0
	14.7	4.87	3.0
	12.2	5.02	3.1

* The value 3.52 in the original is evidently a typographical error.

The $\sqrt{\mu}$ for these solutions, therefore, probably ranges between 0.15 and 0.30. At 38° $\log K'_{\text{equil.}}$ varies from 2.7 to 3.1 in this range; at room temperature it varies also from 2.7 to 3.1. This indicates that the temperature coefficient of $K'_{\text{equil.}}$ is not very large, since there is little difference between the values obtained at room and body temperatures.

There are then forty-eight experiments at 38° and also sixteen

experiments at room temperature in which solutions containing calcium and phosphate were equilibrated with precipitates of calcium phosphate. The solid phase in some instances was secondary calcium phosphate, in others it approximated the composition of tricalcium phosphate, and in others it was more basic than tricalcium phosphate. *It is remarkable that the ratio $[Ca^{++}] : [H^+]$ is a constant regardless of the composition of the solid phase.*

We desire to emphasize that this equilibrium constant K'_{equil} is an empirical constant obtained directly from experimental data and that it involves no assumptions regarding the mechanism of the chemical reactions concerned. This experimental constancy may be written in two other ways. According to Sendroy and Hastings

$$pH = pK_2' + \log \frac{[HPO_4^{=}] }{[H_2PO_4^{-}]} \quad (9)$$

or

$$[H^+] = K_2' \frac{[H_2PO_4^{-}]}{[HPO_4^{=}]} \quad (10)$$

Substituting this value of $[H^+]$ in equation (5) we get

$$\frac{[Ca^{++}] \times [HPO_4^{=}]}{[H_2PO_4^{-}]} = K_2' \times K'_{\text{equil}} \quad (11)$$

at zero ionic strength $K_2' = 10^{-7.15}$ and $K'_{\text{equil}} = 10^{2.4}$. Substituting these values in equation (11) we get

$$K_{\text{equil}} = \frac{[Ca^{++}] \times [HPO_4^{=}]}{[H_2PO_4^{-}]} = 10^{-4.8} \quad (12)$$

From equation (12) it appears that the final equilibrium which is attained depends upon both the ion product of $CaHPO_4$ and hydrolysis of the $[HPO_4^{=}]$ ion to $[H_2PO_4^{-}]$. It is significant that this constant holds not only when the solid phase is $CaHPO_4$, but also when it is tricalcium phosphate and when it is more basic than tricalcium phosphate. This is in accord with the opinion

which we expressed in preceding sections that the precipitate throughout the entire range may contain CaHPO_4 .

In alkaline solutions the Ca:P ratio in the precipitate which forms immediately after mixing is greater than 1.29; in acid solutions under some conditions the initial solid phase with a ratio of 1.29 changes to one which at equilibrium has a ratio approximating 1.94. In what form is this additional calcium? This question cannot be definitely answered at present. It is possible that the solid phase removes calcium from the solution by adsorption. It is also possible that there is a deposition in some way of some basic calcium compound like CaO , or Ca(OH)_2 . It is interesting to note that the experimental statement

$$\frac{[\text{Ca}^{++}]}{[\text{H}^+]} = K'_{\text{equil.}} \quad (5)$$

may be written

$$[\text{Ca}^{++}] \times [\text{OH}^-] = K'_{\text{equil.}} \times 10^{-14} \quad (13)$$

since

$$[\text{H}^+] \times [\text{OH}^-] = k_w = 10^{-14} \quad (14)$$

On multiplying equations (13) and (14) we get

$$([\text{Ca}^{++}] \times [\text{OH}^-]^2) \times [\text{H}^+] = K'_{\text{equil.}} \times 10^{-28} = \underline{\underline{K'_{\text{equil.}}}} \quad (15)$$

At zero ionic strength

$$\underline{\underline{K_{\text{equil.}}}} = ([\text{Ca}^{++}] \times [\text{OH}^-]^2) \times [\text{H}^+] = 10^{-28.6} \quad (16)$$

From this calculation it is seen that when the experimentally obtained constant is multiplied by the square of the ion product of water, *i.e.* by 10^{-28} , another equilibrium constant is obtained. This constant also may have theoretical significance, for it is dependent upon the ion product of Ca(OH)_2 and the hydrogen ion concentration.

Calcification in Vitro.

Shipley, Kramer, and Howland (9) obtained calcification *in vitro* by using solutions containing only inorganic constituents.

We have repeatedly performed experiments on calcification *in vitro* and wish to point out that in such experiments *calcification* is obtained and not *precipitation*; calcium phosphate is deposited in a narrow zone across the epiphyses, exactly analogous to the line test obtained in calcification *in vivo*. It is usually deposited in no other part of the wide ricketic zone, nor is any precipitate formed in the supernatant solution. Calcification may therefore be studied in systems in which the composition of the solution may be varied at will.

Inorganic serum solutions were prepared and the pH was adjusted to 7.3; these solutions resemble serum closely except that

TABLE III.

Ion Product of CaHPO_4 in Inorganic Serum Solutions.

$\sqrt{\mu} = 0.38$, pH = 7.35, Ca = 10.0 mg. per 100 cc.

P per 100 cc.	Ca \times P.	$[\text{Ca}^{++}] \times [\text{HPO}_4^{--}]$.
<i>mg.</i>		
6.0	60	4.0×10^{-6}
5.0	50	3.3×10^{-6}
4.0	40	2.7×10^{-6}
3.5	35	2.3×10^{-6}
3.0	30	2.0×10^{-6}
2.0	20	1.3×10^{-6}
1.5	15	1.0×10^{-6}

Calcification.

No calcification.

$K'_{s.p.} \text{CaHPO}_4 = (3.2 \pm 0.8) \times 10^{-6}$ for $\sqrt{\mu} = 0.38$; i.e., between 2.4×10^{-6} and 4.0×10^{-6} .

they contain no organic material. When these solutions were employed it was found that calcification was obtained when the product Ca \times P exceeded 40; no calcification was obtained when this product was below 30.† We have calculated the ion product of CaHPO_4 in inorganic serum solutions having a constant calcium content of 10 mg. per cent and a phosphorus content ranging from 1.5 to 6.0 mg. per cent. The results are given in Table III.

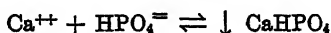
When these ion products are compared with $K'_{s.p.} \text{CaHPO}_4$ for that ionic strength, the striking observation is made that *these ion products are of the same order of magnitude as the solubility*

† This is the empirical product of Howland and Kramer (58) in which the concentrations of Ca and P are expressed in mg. per 100 cc.

product of CaHPO_4 . When $\sqrt{\mu}$ is 0.38, $\text{pK}'_{s.p.} \text{CaHPO}_4 = 5.5$, § with an estimated error of ± 0.1 ; i.e., $K'_{s.p.} \text{CaHPO}_4 = (3.2 \pm 0.8) \times 10^{-6}$. This means that for these solutions the solubility product lies somewhere between 2.4×10^{-6} and 4.0×10^{-6} , while the ion products range from 1.0×10^{-6} to 4.0×10^{-6} . Solutions with a $\text{Ca} \times \text{P}$ product less than 30 are therefore undersaturated with respect to CaHPO_4 . This may account for the fact that calcification *in vitro* cannot be obtained in solutions with a $\text{Ca} \times \text{P}$ product less than 30. Solutions with $\text{Ca} \times \text{P}$ products from 35 to 60 have for the ion product $[\text{Ca}^{++}] \times [\text{HPO}_4^{=}]$ values which are very close to the solubility product of CaHPO_4 . ||

Shipley, Kramer, and Howland have shown that, when the $\text{Ca} \times \text{P}$ product is 35, calcification is obtained *in vitro* not only when the calcium is 10 mg. and the phosphorus is 3.5 mg., but also when the calcium is 5 mg. and the phosphorus is 7 mg. The importance of this empirical $\text{Ca} \times \text{P}$ product was first pointed out by Howland and Kramer (58, 59). They showed that in young children and in young rats when the product of calcium and inorganic phosphorus in serum was below 30, active rickets was present; when the $\text{Ca} \times \text{P}$ product was above 40, rickets was either absent or healing had set in. The dividing value between calcification and absence of calcification was in the neighborhood of 35. They observed healing rickets in one instance when the calcium was 5.5 mg.; the $\text{Ca} \times \text{P}$ product in this case was 44. Thus the empirical $\text{Ca} \times \text{P}$ product applies in the same way to calcification *in vivo* and *in vitro*.

The fact that this empirical product has proved so valuable a guide clinically and experimentally gives rise to the thought that there may be a theoretical basis for it. This empirical product would have theoretical validity if the calcium were deposited as the result of a second order reaction such as



§ These values for $\text{pK}'_{s.p.} \text{CaHPO}_4$ are taken from the curve obtained when the values of $\text{pK}'_{s.p.} \text{CaHPO}_4$ are plotted against $\sqrt{\mu}$. They may also be calculated from equation (3,a), p. 129.

|| The curve of Holt, La Mer, and Chown ((5), Fig. 10) gives a value of 5.6 for $\text{pK}'_{s.p.} \text{CaHPO}_4$ at this ionic strength; i.e., $K'_{s.p.} \text{CaHPO}_4 = 2.5 \times 10^{-6}$. This is the value obtained from their theoretical curve in which $\Delta \text{pK}_{s.p.} = 4\sqrt{\mu}$.

because

$$\text{Ca} \times \text{P} \propto [\text{Ca}^{++}] \times [\text{HPO}_4^{=}]$$

This function is a linear one and is, at the pH of serum,

$$\text{Ca} \times \text{P} = 1.5 \times 10^7 ([\text{Ca}^{++}] \times [\text{HPO}_4^{=}])$$

Effect of pH.—The value of the product $\text{Ca} \times \text{P}$ is limited to the pH of serum; it does not hold in solutions whose reaction differs markedly from a pH of 7.3. Kramer, Shelling, and Orent (60) studied the effect of pH on calcification *in vitro* in this laboratory. Their results show that when the pH is below 7.0, calcification is not obtained even with a product of 50.

TABLE IV.
Inorganic Serum Solutions $\sqrt{\mu} = 0.38$.

Ca per 100 cc.	P per 100 cc.	pH	Ca \times P.	$[\text{Ca}^{++}] \times [\text{HPO}_4^{=}]$.
<i>mg.</i>	<i>mg.</i>			
5.0	7.0	7.35	35	2.3×10^{-6}
7.8	6.5	7.35	51	3.4×10^{-6}
10.0	3.5	6.90	35	1.8×10^{-6}
10.0	3.5	6.80	35	1.7×10^{-6}
10.0	4.0	6.90	40	2.1×10^{-6}
10.0	4.0	6.80	40	1.9×10^{-6}
5.5	8.0	7.35	44	2.9×10^{-6}
5.5	8.0	6.80	44	2.1×10^{-6}

Although the empirical $\text{Ca} \times \text{P}$ product is not applicable in solutions more acid than serum, the theoretical ion product $[\text{Ca}^{++}] \times [\text{HPO}_4^{=}]$ still gives consistent results. These results are summarized in Table IV. It is seen from this table that empirical products from 35 to 44 have ion products of 2.3×10^{-6} or greater when the pH is 7.35; when the pH drops to slightly below 7.0 the ion products are less than 2.3×10^{-6} ; i.e., they are undersaturated with respect to CaHPO_4 even with an empirical $\text{Ca} \times \text{P}$ product of 44. It therefore appears that calcification *in vitro* is not obtained when the ion product $[\text{Ca}^{++}] \times [\text{HPO}_4^{=}]$ is less than 2.3×10^{-6} . This ion product has a wider applicability than the $\text{Ca} \times \text{P}$ product; it may therefore be found to be more

useful than the latter product. In that case, the approximate dividing value between calcification and no calcification may be taken as 2.5×10^{-6} .

Local Factors.—According to the above calculations these inorganic serum solutions at pH 7.3 are undersaturated with respect to CaHPO_4 when their $\text{Ca} \times \text{P}$ products are less than 30. For empirical products between 35 and 60 these solutions are probably very slightly undersaturated or just saturated with respect to CaHPO_4 . This would account for the fact that these solutions are clear. Why then, when sections of ricketic bone are immersed in the latter solutions, should calcification occur? Furthermore, why should calcification take place in a definitely localized area of the sections?

The entire problem of the factors operating locally is still unsolved. It is obvious that these local factors are of importance else calcification would occur throughout the entire uncalcified zone. At the present time we can only venture a guess. It is possible that within the matrix[¶] the pH is greater than 7.3 or that $\sqrt{\mu}$ may be less than 0.38. Either one, or both, of these factors may produce a local supersaturation and consequent precipitation of CaHPO_4 .

If for any reason the inside of the matrix is more alkaline than the solution which bathes it, $[\text{HPO}_4=]$ inside the matrix will be greater than in the solution even though the total $[\text{P}]$ is the same, for $[\text{HPO}_4=]$ will increase at the expense of $[\text{H}_2\text{PO}_4-]$. This increase of $[\text{HPO}_4=]$ may be just sufficient to cause $[\text{Ca}^{++}] \times [\text{HPO}_4=]$ to exceed $K'_{s.p.}$ CaHPO_4 (see Robison and Soames (3)).

If for any reason the matrix fluid has an ionic strength lower than the outside solution, it will have a correspondingly lower value of $K'_{s.p.}$ CaHPO_4 . Thus, for example, a value of 3.0×10^{-6} for the ion product $[\text{Ca}^{++}] \times [\text{HPO}_4=]$ is less than that of the solubility product of CaHPO_4 in a solution with $\sqrt{\mu}$ equal to 0.38, since at that ionic strength $K'_{s.p.}$ CaHPO_4 has a value of 3.2×10^{-6} . The same value of 3.0×10^{-6} for the ion product $[\text{Ca}^{++}] \times [\text{HPO}_4=]$ will be greater than $K'_{s.p.}$ CaHPO_4 in a similar solution

[¶] According to Shipley, Kramer, and Howland (9), the calcium phosphate is deposited in the intercellular matrix, just as is the case when healing of rickets occurs *in vivo*.

differing from the first only in that $\sqrt{\mu}$ is 0.31 instead of 0.38, since at the lower ionic strength $K'_{s.p.}$ CaHPO_4 has a value of 2.0×10^{-6} .** A concentration of $[\text{Ca}^{++}]$ and $[\text{HPO}_4^{=}]$ which gives a slightly undersaturated solution in the outside fluid may therefore give a slightly supersaturated solution with consequent precipitation in the inside fluid.

Calcification in Vivo.

The chief difference between serum and inorganic serum solutions is that serum contains proteins and possibly other substances which "bind" calcium so that the equation $[\text{Ca}] = [\text{Ca}^{++}]$ does not hold for serum. This does not affect the validity of the considerations just presented. Inorganic serum solutions with $\text{Ca} \times \text{P}$ products less than 30 are, according to the above calculations, undersaturated with respect to CaHPO_4 ; serum with similar $\text{Ca} \times \text{P}$ products should therefore also be undersaturated with respect to CaHPO_4 . The ion product $[\text{Ca}^{++}] \times [\text{HPO}_4^{=}]$ in serum with a given calcium and phosphorus content cannot be greater than the ion product in an inorganic serum solution with the same calcium and phosphorus content. If the value of the ion product is different it must be less in serum, the difference depending upon the amount of calcium bound in unionized form. If CaHPO_4 is the substance which deposits from solution, then, according to these calculations, serum is *undersaturated* with respect to calcium phosphate.

The possible rôle of CaHPO_4 was considered by Howland and Kramer (59). Although they believed that bone consisted of tricalcium phosphate, yet they realized that the ion product of CaHPO_4 might be important. They stated: "There must be a solubility product constant for Ca and HPO_4 ions which has a definite value at a given pH and temperature. When this constant is exceeded precipitation must take place. . . . While the ionic concentration cannot be measured we can prove that the general principle holds true by clinical observation using as factors for a rough product the total concentration of calcium and of phosphorus in the serum."

** See foot-note, §, p. 137.

It has not been demonstrated as yet that calcification occurs when the ion product $[Ca^{++}] \times [HPO_4=]$ exceeds $K'_{s.p.} CaHPO_4$. Our calculations show, however, that failure to calcify occurs when this ion product is definitely less than the solubility product of $CaHPO_4$.

That normal serum is very near the saturation point with respect to $CaHPO_4$ is also indicated by an experiment of Mond and Netter (61). These investigators shook serum with $CaHPO_4$ at body temperature. The serum contained 9.4 mg. per cent calcium at the beginning; at the end of an hour the serum calcium was still 9.4 mg. per cent.

CONCLUSION.

We have not discussed the precipitation of $CaCO_3$ nor the rôle of $K'_{s.p.} CaCO_3$. It seems that calcium phosphate is the substance which determines calcification; when conditions are such that calcium phosphate is not deposited, then so far as our present knowledge goes, no bone salts are deposited. It is our present opinion that the rôle of $K'_{s.p.} CaCO_3$ in calcification will not be made clear until the behavior of calcium phosphate is understood.

In conclusion we desire to differentiate sharply between (1) conclusions which we regard as definitely established and (2) theories which we have suggested to account for observed findings. The following résumé includes only those points which we consider as definitely established.

The presence in bones of a compound with the formula $Ca_3(PO_4)_2$ has never been demonstrated; there is no evidence which shows that bone contains calcium and phosphate in even the same empirical ratio as $Ca_3(PO_4)_2$. There are in the literature exceedingly few analyses of unashed bones accurate to 0.5 per cent; these few analyses indicate the presence of basic calcium in bone.

The existence of a substance with the formula $Ca_3(PO_4)_2$ has never been demonstrated, not even in simple inorganic systems. In spite of repeated attempts during the last 100 years no one has succeeded in preparing precipitates with even the empirical composition of $Ca_3(PO_4)_2$.

The ion product $[Ca^{++}]^3 \times [PO_4=]^2$ fails to account satisfactorily for either the formation of the initial precipitate from solution or for the final equilibrium between the solid phase and the super-

natant solution. It also fails to account satisfactorily for bone formation.

Secondary calcium phosphate is a definite chemical entity with well defined crystal forms. At 38° and at zero ionic strength the solubility product of CaHPO_4 is $(4 \pm 1) \times 10^{-7}$. Up to the ionic strength of serum the relation between the stoichiometric solubility product and the ionic strength is an agreement with modern theories of solution.

When final equilibrium is attained between a precipitate of calcium phosphate and its supernatant solution, following prolonged equilibration, it is found that $\frac{[\text{Ca}^{++}]}{[\text{H}^+]}$ is a constant. This empirical equilibrium constant varies with the ionic strength in agreement with modern theories of solution. It is noteworthy that this same constant is obtained regardless of the composition of the solid phase at equilibrium.

Calcification is not obtained *in vitro* when the ion product $[\text{Ca}^{++}] \times [\text{HPO}_4=]$ in the external solution is definitely less than $K'_{s.p.} \text{CaHPO}_4$. Calcification *in vitro* is obtained when this ion product is very nearly equal to $K'_{s.p.} \text{CaHPO}_4$. At a pH of 7.3 this ion product is as applicable as the empirical $\text{Ca} \times \text{P}$ product; moreover it is applicable in more acid solutions where the empirical $\text{Ca} \times \text{P}$ product does not hold.

SUMMARY.

1. As a criterion for the formation of precipitates of calcium phosphates, the ion product $[\text{Ca}^{++}]^3 \times [\text{PO}_4=]^2$ is inadequate.

2. We have found no evidence which satisfactorily demonstrates the presence in bones of a compound with the formula $\text{Ca}_3(\text{PO}_4)_2$.

3. From data in the literature $\text{p}K_{s.p.} \text{CaHPO}_4$ is calculated to be 6.4 ± 0.1 at 38° . At any ionic strength up to that of serum

$$\text{p}K'_{s.p.} \text{CaHPO}_4 = 6.4 - 2.3 \sqrt{\mu}$$

4. The ion product $[\text{Ca}^{++}] \times [\text{HPO}_4=]$ at which calcification begins, both *in vivo* and *in vitro*, is numerically equal to the value calculated for $K'_{s.p.} \text{CaHPO}_4$ at the ionic strength of serum.

5. A theoretical basis is suggested for the empirical $\text{Ca} \times \text{P}$ product. In inorganic serum solutions with a pH of 7.35

$$\text{Ca} \times \text{P} = 1.5 \times 10^7 ([\text{Ca}^{++}] \times [\text{HPO}_4^{=}])$$

6. Inorganic serum solutions with empirical $\text{Ca} \times \text{P}$ products less than 30 appear to be *undersaturated* with respect to CaHPO_4 ; calcification is not obtained with these solutions.

7. The evidence indicates that ricketic serum is definitely undersaturated with respect to CaHPO_4 and that normal serum is slightly undersaturated with respect to this salt.

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COMPOSITION OF BONE.

IV. PRIMARY CALCIFICATION.*

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The chemistry of the calcium phosphates together with its bearing on calcification was discussed in the preceding communication (1). The evidence indicated that the composition of bone when first deposited might be different from the composition of old bone. In inorganic aqueous solutions the primary precipitate of CaHPO_4 with a Ca : P ratio of 1.29 undergoes transformation, in many cases, to a solid phase in which Ca : P approximates 1.94. Similarly, although in bone the ratio Ca : P also approximates 1.94, it seemed possible that this ratio might be different in freshly deposited bone.

This secondary reaction, which precipitates of calcium phosphate undergo, is completed in about 8 days in inorganic solutions as Holt, La Mer, and Chown (2) have shown. If a similar reaction occurs *in vivo* its detection would require that analysis be made on bone that is not more than a few days old. We have utilized for such analysis the fresh calcification deposited in ricketic rats whose rickets had just begun to heal. The analytical procedures employed were those described in the first paper of this series (3).

Normal Rat Bone.

Sixteen specimens of bone from normal rats were analyzed with the micro technique. Calcium, phosphate, and CO_2 were determined in duplicate on each specimen. The analytical results are given in Table I. It is seen from this table that the ratio residual

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TABLE I.
Normal rat.

Age.	Bones.	Ca	P	CO ₂	Carbonate Ca Total Ca × 100.	Residual Ca P	Deviation.
<i>days</i>		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>			
1	All.	13.7	6.4	1.23			
		13.2	6.5	1.32	9	1.89	-0.10
5	Leg.	15.8	7.2	1.62			
		15.9	7.5	1.61	9	1.95	-0.04
5	Rib.	11.5	5.4	1.24			
		12.1	5.4	1.31	10	1.96	-0.03
5	Head.	14.6	6.7	1.62			
		14.5	6.7	1.54	10	1.97	-0.02
13	Leg.	12.4	5.7	1.12			
		12.4	5.7	1.16	8	2.00	+0.01
15	"	11.4	5.6	1.36			
		12.3	5.7	1.22	10	1.88	-0.11
15	Rib.	11.6	5.3	1.14			
		11.6	5.2	1.03	9	2.00	+0.01
20	Leg.	13.4	6.4	1.30			
		13.2	6.2	1.28	9	1.92	-0.07
20	Rib.	11.5	5.1	1.06			
		11.7	5.1	1.09	8	2.08	+0.09
38	Leg.	13.9	5.8	1.61			
		13.9	5.9	1.54	10	2.12	+0.13
38	Rib.	13.2	6.1	1.18			
		13.0	5.9	1.20	8	2.00	+0.01
46	Leg.	18.8	7.8	2.57			
		18.6	7.8	2.49	12	2.10	+0.11
63	Shafts.	21.8	9.3	3.20			
		21.7	9.2	3.19	13	2.03	+0.04
5 mos.	Leg.	21.0	8.9	3.62			
		21.7	8.8	3.99	16	2.01	+0.02
Adult 1.	"	24.1	10.3	4.05			
		23.9	10.2	4.16	16	1.97	-0.02
" 2.	"	23.5	10.1	3.79			
		23.4	10.2	3.86	15	1.96	-0.03
Mean = 1.99						a.d. = ±0.05 A.D. = ±0.01	

Residual Ca: P = 1.99 ± 0.01.

Ca : P obtained with the micro technique has a mean value of 1.99 for normal rat bone. The average deviation from the mean, *a.d.*, is ± 0.05 , and *A.D.* = ± 0.01 . The mean value of 1.99 ± 0.01 obtained with the micro technique is in agreement with similar results obtained by others using macro methods.

The values in the column entitled $\frac{\text{carbonate Ca}}{\text{total Ca}} \times 100$ were obtained by means of the expression

$$\frac{\text{Per cent CO}_2 \times 91}{\text{Per cent Ca}} = \frac{\text{carbonate Ca}}{\text{total Ca}} \times 100$$

The per cent of CO₂ multiplied by 0.91 gives the per cent of calcium assumed to be present as bound to carbonate in CaCO₃; *i.e.*, this gives the per cent of carbonate calcium. Dividing per cent of carbonate calcium by per cent of total calcium and multiplying by 100 gives the values in this column.

It is seen from this column that the bones of very young rats contain less CaCO₃ in proportion to calcium phosphate than do bones of older rats. In young rats carbonate Ca constitutes only 8 to 10 per cent of the total Ca, while in adult rats it constitutes about 15 per cent. This means that in the bones of rats the proportion of CaCO₃ to the other calcium salts increases markedly with age. Transition values were obtained in rats 6 and 9 weeks old.

This difference in the carbonate content of the bones of young and old rats is not due to experimental error. If the CO₂ values in young rats were too low, or if the values of total Ca were too high, then these errors would be reflected in the ratio residual Ca : P. All the bones, from rats varying in age from 1 day to maturity, gave the same value within the experimental deviation for the ratio residual Ca : P; the change in carbonate content with increasing age is, therefore, a real one.

The analyses and ratios given in Table I are selected values. Some of these bones were analyzed in duplicate and some in triplicate; in one case five analyses were made of the same bone. All of these analyses were performed as described previously—the calcium, phosphorus, and CO₂ in each case were determined at least in duplicate. The ratios obtained in all of these analyses

TABLE II.
Normal Rat.

Summary of all ratios.

Age.	Bones.	Ratio.	Deviation.
<i>days</i>			
1 and 2	All.	1.89	-0.12
5	Leg.	1.95	-0.06
5	Rib.	1.96	-0.05
5	Head.	1.97	-0.04
		1.97	-0.04
		1.90	-0.11
13	Leg.	2.10	+0.09
		2.00	-0.01
15	"	1.88	-0.13
15	Rib.	2.12	+0.11
		2.00	-0.01
		2.02	+0.01
20	Leg.	1.94	-0.07
		2.05	+0.04
		1.92	-0.09
20	Rib.	2.08	+0.07
38	Leg.	1.93	-0.08
		2.12	+0.11
		2.12	+0.11
38	Rib.	2.21	+0.20
		2.02	+0.01
		2.00	-0.01
46	Leg.	2.14	+0.13
		2.10	+0.09
63	Shafts.	2.03	+0.02
5 mos.	Leg.	2.01	0.00
Adult 1.	"	1.98	-0.03
		1.99	-0.02
		2.00	-0.01
		1.97	-0.04
		2.04	+0.03
" 2.	"	2.04	+0.03
		2.03	+0.02
		1.96	-0.05
		1.98	-0.03
		Mean = 2.01	<i>a.d.</i> = ± 0.06 <i>A.D.</i> = ± 0.01

Residual Ca: P = 2.01 ± 0.01 .

are given in Table II. These ratios have all been given here and have all been included in calculating the mean and the *A.D.*, for the sake of comparison with the ratios given in Table VI. The value 2.21 for example is obviously erroneous (two subsequent analyses of the same bone gave ratios of 2.02 and 2.00); it has nevertheless been included. The mean obtained from *all* the analyses of normal rat bone is 2.01 ± 0.01 .

TABLE III.
Primary Calcification (Rat).

Healing induced with cod liver oil concentrate.

Bone.	No. of samples ana- lyzed.	Ca	P	CO ₂	$\frac{\text{Carbonate Ca}}{\text{Total Ca}} \times 100.$	$\frac{\text{Residual Ca}}{\text{P}}$
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>		
Fresh calcifica- tion (lines).	4	7.8	3.1			
		7.5	3.0			
		7.9	2.9			
		7.6	2.9			
		7.7	2.8	0.89		
			2.9	0.93	11	2.38
			2.9	0.91		
Epiphyses and diaphyses.	3	10.1				
		9.9	4.2	1.46		
		10.2	4.1	1.43	13	2.10
		10.1	4.2	1.45		
Old bone (shafts).	1	15.6	6.4	2.78		
		15.5	6.3	2.61	16	2.05
		15.6	6.4	2.70		

Primary Calcification.

Rickets was produced in rats by feeding Steenbock's Ration 2965.¹ Severe rickets was obtained in 4 weeks; sections from the upper end of the tibia showed a wide layer of uncalcified cartilage and a wide metaphysis. Twenty-five of these rats were fed cod liver oil concentrate for 8 days. Autopsy showed that the rickets had begun to heal; there was a line of fresh calcification in the provisional zone of cartilage.

¹ For the composition of Ration 2965 see Steenbock, H., and Black, A., *J. Biol. Chem.*, 1925, lxiv, 263.

All the rats were killed, and the upper end of each tibia was removed and sectioned. Each section contained the center of ossification, the epiphyseal cartilage, the line of fresh calcification, the metaphysis, and a little of the shaft. Each section was cut in three places at right angles to the axis of the shaft; the different portions of the sections were combined in three groups and labelled "shafts," "epiphyses and diaphyses," and "lines." Each of these three portions of the upper end of the tibia was then analyzed by means of the micro technique. The results are given in Table III.

In each case calcium, phosphate, and CO_2 were determined at least in duplicate. In the case of the new bone from the lines there were four analyses for calcium and six analyses for phosphate. Separate samples were digested for the additional analyses; they do not represent merely aliquots of the same solution. It is seen that the shafts gave a ratio of 2.05; this is within the normal range. The epiphyses and diaphyses gave a ratio of 2.10; this is somewhat high, probably because there was some fresh calcification present in addition to the older bone. The fresh calcification of the lines gave a decidedly high ratio of 2.38.

A similar study was carried out on another set of ricketic rats except that irradiated yeast was employed as the antiricketic agent. In these rats the healing of the rickets was not confined to the test line, as there was also considerable fresh calcification in the metaphysis itself. The sections of the tibia were therefore divided into three groups somewhat different from those in the preceding set of rats; these groups were "lines," "metaphyses," and "shafts." The results are given in Table IV.

Table IV shows that the old bone from the shafts gave a ratio of 2.05; this is within the normal range. The metaphyses gave a high ratio of 2.25 and the lines gave a moderately high ratio of 2.16. In analyzing a normal bone it sometimes happens that two errors occur in which a high calcium value and a low phosphorus value are obtained simultaneously. This would result in a high Ca:P ratio. To rule out this possibility ten analyses were made for calcium and twelve for phosphorus. A number of samples of the material were digested and analyzed separately; the value 2.16 is calculated from the mean values so obtained.

A third study was made in which the healing of the rickets was induced by butter. To the ricketic diet was added 1 per cent but-

ter; this mixture was fed for 4 weeks. At the end of this period autopsy showed that there was fresh calcification in the metaphysis but not in the provisional zone. The metaphyses were removed as described above, and analyzed. The shafts were also analyzed. The results are given in Table V.

TABLE IV.
Primary Calcification (Rat).

Healing induced with irradiated yeast.

Bone.	Ca	P	CO ₂	$\frac{\text{Carbonate Ca}}{\text{Total Ca}} \times 100.$	$\frac{\text{Residual Ca}}{\text{P}}$
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>		
Fresh calcification (lines).	12.8	4.9			
	12.3	4.9			
	12.3	5.4			
	12.2	5.2			
	12.2	5.3			
	12.6	5.2			
	12.4	5.2			
	12.3	5.2			
	12.4	5.1			
	12.6	5.1			
	12.4	5.1	1.67		
		5.1	1.51	12	2.16
		5.1	1.59		
Fresh calcification (metaphyses).	11.7				
	12.2	4.6			
	11.9	4.6			
	12.2	4.8	1.64		
	12.1	4.9	1.48	12	2.25
	12.0	4.7	1.56		
Old bone (shafts).	20.4	8.5			
	20.7	8.5			
	20.7	8.5	3.43		
	20.8	8.7	3.41	15	2.05
	20.7	8.6	3.42		

The old bone in the shafts gave a normal ratio of 1.94; the new bone in the metaphyses gave a high ratio of 2.14. To exclude the possibility of this high ratio being due to experimental error, repeated analyses were made for calcium and phosphorus. The ratio in the last column was calculated from the mean values.

In another group of ricketic rats healing was induced by irradiated cholesterol. The deposition of fresh calcification occurred in the provisional zone of cartilage; the lines were removed and analyzed. The results are given in Table VI. It is seen that this gave a high Ca : P ratio of 2.24. For comparison the legshafts of a number of normal rats of the same age, *i.e.* 63 days old, were removed and analyzed. A normal ratio of 2.03 was obtained.

TABLE V.

Primary Calcification (Rat).

Healing induced with butter.

Bone.	Ca	P	CO ₂	$\frac{\text{Carbonate Ca}}{\text{Total Ca}} \times 100.$	$\frac{\text{Residual Ca.}}{\text{P}}$
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>		
Fresh calcification (metaphyses).	10.0	4.4			
	10.1	4.2			
	10.4	4.2			
	10.1	4.2			
	10.2	4.2			
	10.2	4.6			
	10.2	4.5			
		4.4			
		4.4			
		4.3	1.10		
Old bone (shafts).		4.3	1.11	10	2.14
		4.3	1.11		
	20.3	8.9			
	19.8	8.5			
	20.1	9.0	3.32		
		8.7	3.26	15	1.94
		8.8	3.29		

DISCUSSION.

In interpreting the results of analyses of bones it is customary to assume that all the CO₂ is present as CaCO₃ and that all the Mg is present as Mg₃(PO₄)₂. The carbonate calcium is deducted from the total calcium, and the phosphate equivalent to the magnesium is deducted from the total phosphate. It has been repeatedly found that the residual calcium and residual phosphate so obtained give a ratio of Ca : P which approximates that calcu-

lated from the formula $\text{Ca}_3(\text{PO}_4)_2$. When analyzed with our micro technique, normal rat bone gives a ratio for residual Ca : P which approximates 1.94.² The Ca : P ratios which we have obtained for normal rat bone by means of the micro technique are therefore in agreement with the results of previous investigators.

Proportion of CaCO_3 .—Our analyses show that the proportion of CaCO_3 to calcium phosphate in bone increases with the age of

TABLE VI.
Primary Calcification (Rat).

Healing induced with irradiated cholesterol.

Bone.	Ca	P	CO_2	$\frac{\text{Carbonate Ca}}{\text{Total Ca}} \times 100.$	$\frac{\text{Residual Ca}}{\text{P}}$
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>		
Fresh calcification (lines).	9.9	3.8			
	9.9	3.8			
	9.3	3.6			
	9.0	3.6			
	9.0	3.6			
	9.2	3.9			
	9.1	3.8			
	9.2	3.8			
	9.2	3.8			
	9.3	3.7			
		3.6	1.12		
		3.8	1.16	11	2.24
		3.7	1.14		
Old bone (shafts of normal rats 63 days old).	9.3	9.3			
	21.8	9.3	3.20		
	21.7	9.2	3.19	13	2.03
	21.8	9.3	3.20		

the animal. There are no recent analyses of unashed bones with which we may compare these results.

This question has apparently not been studied within the last 35 years; we have found no recent analyses, either of ashed or of unashed bone, which have bearing on this point. In 1855 Frémy (4) concluded that in humans there is, with increasing age, a relative increase of CaCO_3 with respect to

² The effect of the omission of the magnesium analysis is discussed in the first paper (3).

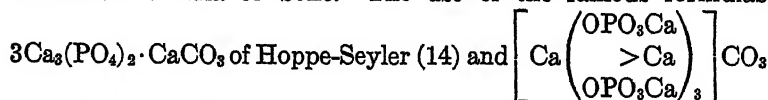
calcium phosphate. Von Recklinghausen (5) in 1858 concluded from his own analyses and from the work of Bibra (1844), of Stark (1845), of Heintz (1849), and of Frémy, that there was no observable difference in composition between the bones of young and those of old individuals. In 1860 Milne Edwards (6) reported that his own analyses agreed with those of Bibra and of Frémy in showing that the proportion of carbonate in the bones of young animals was smaller than in adult bone. Zalesky (7) in 1866 reviewed the literature and concluded that the bones of children of various ages have the same composition as those of adults, not only as a whole, but also as regards the proportion of individual constituents. He pointed out that the methods of determining CO_2 employed by previous investigators were defective. In 1872 Wildt (8) analyzed³ the ashed bones of rabbits of different ages and found an increase in the proportion of CO_2 with increasing age.

Weiske (10) in 1889 analyzed the ashed bones of birds and found an increase in CaO and CO_2 with advancing age, while the P_2O_5 remained constant. Graffenberger (11) in 1891 analyzed the ashed bones of rabbits and also found an increase of the ratio calcium carbonate: calcium phosphate with increasing age.

The only analyses of unashed bone performed during the last 30 years with which we are acquainted are the few analyses of Gassmann (12) and those of Howland, Marriott, and Kramer (13). The results of the latter investigators show that the ratio of calcium phosphate to calcium carbonate is not necessarily constant; this proportion is different in ricketic and normal bones, and is different in normal human bone as compared with normal rat bone. The effect of age on this ratio was not studied.

Our findings are in agreement with those of Howland, Marriott, and Kramer as regards the increase in the proportion of CaCO_3 in rickets. From our tables it is seen that for normal rats approximately 2 months old, carbonate Ca :total Ca is about 13 per cent; for ricketic rats of the same age it is 15 and 16 per cent, the same as in normal adult rats.

Since the proportions of the inorganic constituents vary also with age, as we have just demonstrated, it is incorrect to assign a definite formula to bone. The use of the famous formulas



of Werner (15) should therefore be discontinued as incorrect and misleading.⁴

³ The data of Wildt and of Weiske are also given by Forbes and Keith (9).

⁴ De Jong has attempted to determine the nature of the "compound"

Primary Calcification.—The results of our analyses of fresh calcification are summarized in Table VII. It is seen that in freshly deposited bone salts the proportion of CaCO_3 is low, carbonate Ca : total Ca being about 11 per cent, while in the older bones of these same animals carbonate Ca : total Ca has a value of 15 to 16 per cent. This shows the same phenomenon as do the data in Table I; *i.e.*, that new bone contains a smaller proportion of CaCO_3 to total calcium than does older bone.

TABLE VII.
Primary Calcification (Rat).

Specimen.	Antirickettic factor.	$\frac{\text{Carbonate Ca}}{\text{Total Ca}} \times 100.$	$\frac{\text{Residual Ca}}{\text{P}}.$	Deviation.
Test lines.	Cod liver oil concentrate.	11	2.38	+0.15
Metaphyses.	Butter.	10	2.14	-0.09
Test lines.	Irradiated cholesterol.	11	2.24	+0.01
" "	Irradiated yeast.	12	2.16	-0.07
Metaphyses.	" "	12	2.25	+0.02
		11	Mean = 2.23	<i>a.d.</i> = ± 0.07 <i>A.D.</i> = ± 0.03

Primary calcification, residual Ca : P = 2.23 ± 0.03 .

Old bone " " " = 1.99 ± 0.01 .

The ratio residual Ca : P obtained for primary calcification is 2.23 ± 0.03 ; for older bones analyzed with the same technique this ratio has the value 1.99 ± 0.01 . At the present time there

in bone by means of x-ray analysis on the basis that "The ratio $\frac{\text{CaCO}_3}{\text{Ca}_3(\text{PO}_4)_2}$ being found about the same everywhere it may readily be suspected that bones are built up chiefly by one compound." He employs the formula $3\text{Ca}_3(\text{PO}_4)_2 \cdot \text{CaX}_2$ (*Proc. Acad. Sc. Amsterdam*, 1926, xxix, 870).

appear to be several ways of interpreting this high Ca : P ratio in primary calcification. They are: (1) experimental error, (2) presence of an acid-soluble organic calcium compound, (3) presence of inorganic calcium in cartilage, and (4) presence of an insoluble basic calcium salt.

The results do not appear to be ascribable to experimental error. Five different specimens were analyzed. The analyses were made repeatedly, on separate samples of each specimen, thus ruling out the simultaneous occurrence of an accidental high calcium value and of an accidental low phosphorus value. In the second place, the ratios for fresh calcification are all high; if the deviations were due to experimental error, some ratios should be below normal. In the third place, the occurrence of ratios as high as 2.20 is quite rare when due to experimental error; only one such ratio was obtained in forty-four analyses, as is seen from Table VI of Paper I of this series. Primary calcification gave a ratio of 2.23 ± 0.03 as compared with 1.99 ± 0.01 for normal rat bone. When all the ratios obtained in analyses of normal rat bone were averaged, including even those shown by subsequent analyses to be too high, a mean value of 2.01 ± 0.01 was obtained. When 2.23 ± 0.03 is compared with 2.01 ± 0.01 it is seen that the accidental occurrence of high ratios does not account for the value obtained for primary calcification.

It is possible that the material analyzed for primary calcification contains an acid-soluble organic calcium compound. However, until the presence of such a compound is demonstrated it is simpler to assume that the apparent excess of calcium is present as inorganic calcium.

The cartilage itself may contain some inorganic calcium aside from the calcium due to the presence of bone salts. Thus even if the ratio of Ca : P in freshly deposited bone salts has a normal value of 1.94, the presence of additional inorganic calcium in the cartilage itself would increase the value of the ratio. At the present time, however, the simplest interpretation of the experimental findings is that the high Ca : P ratio is due to the presence of basic calcium in the freshly deposited bone salts.

From the considerations discussed in the preceding communication we had expected that primary calcification would consist of CaHPO_4 , and would be found to give *low* ratios. Analysis,

however, gave *high* ratios. On reconsidering the chemistry of the calcium phosphates we noted that CaHPO_4 is obtained as a precipitate only from acid solution. Cameron and Seidell (16) found that the precipitate was CaHPO_4 when the supernatant solution was acid, and that when the solution was alkaline the precipitate always contained a larger proportion of lime than is required for $\text{Ca}_3(\text{PO}_4)_2$. Cameron and Bell (17) advanced the view that the solid phases were solid solutions whose composition varied from that of CaHPO_4 to that of pure $\text{Ca}(\text{OH})_2$ and that the solid phase which is usually given as $\text{Ca}_3(\text{PO}_4)_2$ may be a mixture of CaHPO_4 and $\text{Ca}(\text{OH})_2$. They suggested that there might be "only one series extending over the whole range in which the solids are di-calcium phosphate and lime."

Serum has an alkaline reaction; the tissue fluid from which the bone salts are deposited may likewise be alkaline. If this is the case, then a simultaneous deposition of CaHPO_4 and basic calcium might be expected on the basis of analogy to inorganic systems. This would account for the apparent significance of the ion product $[\text{Ca}^{++}] \times [\text{HPO}_4=]$ in clinical and experimental calcification as discussed in the preceding paper, and also for the presence of high Ca : P ratios in primary calcification.

The authors make the following acknowledgments: The cod liver oil concentrate was supplied by the H. A. Metz Research Laboratories; the mercury quartz lamps were loaned by the Hano-via Chemical and Manufacturing Company; the yeast was supplied by the Fleischmann Company. Dr. W. B. Lachenschmid, staff veterinarian, helped in the care and feeding of the animals and, together with Dr. D. H. Shelling, assisted in the dissection of some of the material. Mr. Philip Wilchins gave technical assistance in the chemical analyses.

SUMMARY.

1. When analyzed with the micro technique, unashed normal rat bone gave a value of 1.99 ± 0.01 for the ratio residual Ca:P; this is in agreement with the results of analyses of large amounts of bone by macro methods.
2. The proportion of carbonate in normal rat bone increases with age. The ratio carbonate Ca : total Ca is about 8 to 10 per

cent in the bones of young rats and 15 to 16 per cent in those of adult rats.

3. The proportion of carbonate in ricketic rat bone is greater than in normal rat bone of the same age.

4. The proportion of carbonate in primary calcification is less than in the older bones of the same animals.

5. The ratio residual Ca : P in primary calcification has a high value of 2.23 ± 0.03 . This high ratio is interpreted as indicating the presence of a basic calcium salt in freshly deposited bone salts.

6. The composition of the primary calcification appears to be independent of the antiricketic reagent; high Ca : P ratios were obtained regardless of whether the fresh calcification was induced by cod liver oil concentrate, butter, irradiated cholesterol, or irradiated yeast.

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COMPOSITION OF BONE.

V. SOME PROPERTIES OF CALCIUM CITRATE.*

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The deposition of bone salts appears to be dependent upon physicochemical equilibria existing in serum. Calcium ion plays an important part in these equilibria and it is important to know what proportion of the total serum calcium is present as calcium ion. It is generally agreed that a large part of the serum calcium is bound in non-diffusible compounds by proteins. Moreover the opinion has been advanced by Greenwald (1) and supported by Klinker (2) that an additional part of the total calcium is present in serum in the form of a slightly ionized, diffusible organic calcium compound resembling calcium citrate. Sendroy and Hastings (3) also believe that there is strong evidence for the existence of a slightly ionized, diffusible calcium compound in serum. They suggested that this substance may be an organic compound or some slightly dissociable phosphate compound and pointed out the similarity between the parathyroid hormone and citrate solutions as regards their apparent ability to hold calcium in solution. The ultrafiltration experiments of Shelling and Maslow (4) also point to the formation of an unionized ultrafiltrable calcium citrate compound. These authors found that sodium citrate, when injected into the living animal or added to serum *in vitro*, increased markedly the per cent of ultrafiltrable calcium.

The rôle of citrate, and of substances that resemble citrate in their behavior towards calcium ions, may be an important one in calcification. Thus Shipley, Kramer, and Howland (5) found

* These experiments were reported before the Society for Experimental Biology and Medicine, March 16, 1927 (*Proc. Soc. Exp. Biol. and Med.*, 1927, xxiv, 624.)

that citrate inhibited calcification *in vitro* while lactate and acetate did not. Hastings, Murray, and Sendroy (6) studied the solubility of CaCO_3 in solutions of sodium citrate and concluded that the evidence seemed to indicate the formation of a slightly ionized calcium citrate compound. They also concluded that the parathyroid hormone seems to act towards calcium like citric acid. Although there is a considerable amount of indirect evidence which points to the binding of calcium by citrate to form a diffusible, unionized or slightly ionized substance, there is so far as we are aware no direct evidence. In this paper we are presenting conductivity measurements which give direct evidence for the binding of calcium by citrate.

Solubility of Calcium Citrate.

Our plan had been to make a concentrated solution of calcium citrate, and to follow the change in ionization with dilution by means of conductance measurements. This plan was abandoned because of peculiarities encountered in dissolving calcium citrate in water.

The solubility of $\text{Ca}_3(\text{C}_6\text{H}_5\text{O}_7)_2 \cdot 4\text{H}_2\text{O}$ is given (7) as 0.08496 gm. per 100 gm. of water at 18° and 0.0959 gm. per 100 gm. of water at 25° . We first attempted to dissolve 0.048 gm. of commercial calcium citrate¹ in 100 gm. of water, but the material did not dissolve completely; a considerable portion remained undissolved. An equal volume of water was added, but still solution was incomplete. We then took a smaller amount of the material, using first 0.024 gm. and then 0.011 gm. of calcium citrate per 100 cc. of water. The latter is about one-ninth the amount given as the solubility at 25° , but it did not dissolve, even on boiling. Protracted boiling for several hours finally caused all of the solid material to enter the liquid phase. Another sample, after standing on a hot plate for several days, also finally dissolved completely.

We then attempted to prepare calcium citrate by precipitation and encountered interesting colloidal phenomena. It is peculiar that a salt, which is said to increase in solubility with rise in temperature, is precipitated from solution by boiling. Thus Peterson, Wilson, McCoy, and Fred (8) prepared calcium citrate

¹ Two different specimens of calcium citrate, purchased from different sources, were used in these solubility experiments.

by mixing solutions of citric acid and lime water, "filtering and boiling for a few minutes to precipitate the calcium citrate."

Our experiences indicated that the preparation of solid calcium citrate may involve colloidal coagulation instead of simple precipitation, and that solution of the solid material may involve peptization rather than simple solution. There is another possible explanation for the fact that prolonged boiling caused the calcium citrate to dissolve; it may be that hydrolysis of the dissolved calcium citrate occurs, and that this hydrolysis results in solution of additional salt from the solid phase.

We did not investigate these peculiarities further since our primary purpose was to study the behavior of calcium ions in the presence of citrate ions in solution and not the equilibria existing between solutions of calcium citrate and the solid salt.² We therefore decided to investigate the behavior of the ions by means of conductivity titrations.

Conductivity Titrations.

The procedure employed in these electrical conductivity titrations consists essentially of the determination of the resistance of each of two solutions, and of mixtures of these two solutions in varying proportions.

The measurements were made at $25.0^{\circ} \pm 0.1^{\circ}$ with a cylindrical glass vessel about 2 cm. in diameter and removable electrodes of the Ostwald type. A Wheatstone bridge arrangement was used, in which a Kohlrausch slide wire bridge and a resistance box were employed. The measuring instruments were made by Leeds and Northrup. Three solutions were prepared; 0.00125 N NaCl, 0.005 N sodium citrate, and 0.005 N CaCl_2 .

The CaCl_2 solution was made by decomposing a calculated weight of CaCO_3 with a small volume of HCl. When the carbonate had all dissolved, the solution was boiled vigorously to expel dissolved CO_2 . It was then cooled and diluted. Before being made up to volume the pH was adjusted to 7.3 by means of 0.1 N

² It may, however, be mentioned that the direct precipitation of a substance with the formula $\text{Ca}_3(\text{C}_6\text{H}_5\text{O}_7)_2$ would require a fifth order reaction. Further investigation may reveal interesting similarities between tricalcium citrate and tricalcium phosphate. (Cf. Paper III in this series.)

NaOH with phenol red as indicator. The sodium chloride when dissolved in water gave an acid reaction,³ before being made up to volume the pH was adjusted to 7.3 as in the case of the CaCl_2 . The sodium citrate was found to require very little addition of 0.1 N HCl for attainment of pH 7.3. In making up the solution the formula $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 5\frac{1}{2}\text{H}_2\text{O}$ or $2\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 11\text{H}_2\text{O}$ was employed.

The experimental data are given in terms of observed resistances. The same cell was used throughout this investigation; the specific resistance in each case may therefore be calculated from the observed resistance and the cell constant, which had a value of 0.519.

TABLE I.

Conductivity Titration of 5.0 Cc. of 0.00125 N NaCl with 0.005 N Sodium Citrate.

Na citrate added. (1)	Total volume. (2)	$R_{\text{obs.}}$ (3)	$R_{\text{calc.}}^*$ (4)
cc.	cc.	ohms	ohms
0.0	5.0	848	
0.5	5.5	848	853
1.0	6.0	851	857
1.5	6.5	853	860
2.0	7.0	854	863
3.0	8.0	857	867
4.0	9.0	860	871
5.0	10.0	861	874

Observed resistance of the sodium citrate solution = 899 ohms.

$$*R_{\text{calc.}} = \frac{v_1 R_1 + v_2 R_2}{v_1 + v_2}.$$

Normal Conductivity Titration.—The measured resistance of the NaCl solution was 848 ohms; that of the sodium citrate solution was 899 ohms. 5 cc. of the NaCl solution were placed in the cell and then 0.5 cc. of the sodium citrate solution was added. The mixture was stirred and the resistance of the mixture determined. Another 0.5 cc. of the sodium citrate solution was then added, and the resistance again measured after the mixture was stirred. The addition of aliquots of the sodium citrate solution and the measurement of the resistances of the resulting mixtures were

³In subsequent experiments which we have performed at 38° we have used fused NaCl. The fused salt gives a neutral solution in water.

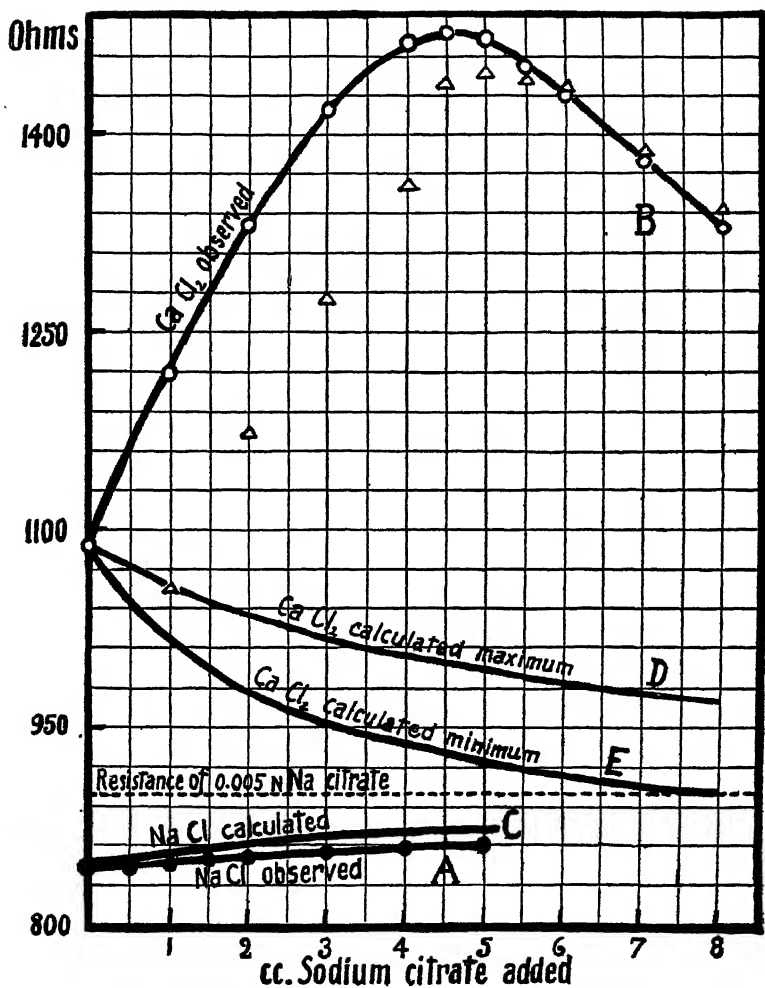


Fig. 1. Conductivity titrations of 0.00125 N NaCl (●) with 0.005 N Na citrate and of 0.005 N CaCl₂ (○) with 0.005 N Na citrate. Δ signifies point to point calculation.

continued until the total volume was 10.0 cc.; *i.e.*, until 5.0 cc. of sodium citrate had been added to the original 5.0 cc. of NaCl. The data are given in Table I and are shown graphically by Curve A in Fig. 1. These values are normal values; *i.e.*, the resistances of the mixtures are intermediate between those of the two original solutions, the resistance of each mixture depending upon the relative quantities of the original solutions in that mixture.

Abnormal Curve.—This same procedure was repeated with 0.005 N CaCl_2 instead of NaCl. The resistance of the CaCl_2 solution

TABLE II.

Conductivity Titration of 5.0 Cc. of 0.005 N CaCl_2 with 0.005 N Sodium Citrate.

Na citrate added.	Total volume.	$R_{\text{obs.}}$	Calculated resistance.			(3) - (6)
			Maximum.	Minimum.	Point to point.	
(1)	(2)	(3)	(4)	(5)	(6)	(3) - (6)
cc.	cc.	ohms	ohms	ohms	ohms	ohms
0.0	5.0	1089	1089	1089	1089	
1.0	6.0	1220	1057	1014	1057	+163
2.0	7.0	1332	1035	979	1174	+158
3.0	8.0	1418	1018	955	1278	+140
4.0	9.0	1468	1005	937	1360	+108
4.5	9.5	1474	999		1438	+ 36
5.0	10.0	1470	994	925	1445	+ 25
5.5	10.5	1454	989		1443	+ 11
6.0	11.0	1428	985	914	1429	- 1
7.0	12.0	1380	978	905	1384	- 4
8.0	13.0	1330	972	899	1343	- 13

$R_{\text{obs.}}$ of 0.005 N Na citrate = 899 ohms.

was 1089 ohms, and that of the 0.005 N sodium citrate solution was 899 ohms. 5 cc. of the CaCl_2 solution were placed in the cell and the resistance measured; it was 1089 ohms. Then 1 cc. of the sodium citrate solution was added; the resistance after this addition was 1220 ohms. Addition of another cc. of sodium citrate caused the resistance to change to 1332 ohms, *etc.* The data are given in Table II and are shown graphically by Curve B in Fig. 1.

The resistance of the citrate solution is less than that of the CaCl_2 solution. On addition of sodium citrate to the latter the resistance of the resulting mixture should be *lower* than that of the CaCl_2 ; it should be intermediate between those of the two

solutions mixed. It is seen, however, that the resistance of the mixture is *higher* than that of the CaCl_2 , and it continues to increase until approximately an equal volume of the citrate solution has been added. After about an equal volume of sodium citrate has been added, then and then only, does the resistance decrease as expected.

The concentration of 0.005 N Ca^{++} was selected because this is the concentration of calcium in normal serum, *i.e.* 10 mg. per 100 cc. This behavior with citrate is, however, not confined to this concentration of sodium citrate. A similar conductivity titration was performed with $\frac{N}{167} \left(\frac{M}{500} \right)$ sodium citrate; a similar curve was obtained showing a marked increase in resistance to a maximum at about 4 cc. of added sodium citrate, followed by a decrease in resistance on further addition of citrate solution.

The same type of curve was also obtained when 0.02 N CaCl_2 was titrated with $\frac{N}{33} \left(\frac{M}{100} \right)$ sodium citrate. The experiments with these various concentrations were performed repeatedly; similar results were obtained in all cases.

Calculated Titration Curves.—The resistance of a mixture of two solutions with resistances R_1 and R_2 respectively, was calculated from the expression

$$R_{\text{mixt.}} = \frac{v_1 R_1 + v_2 R_2}{v_1 + v_2} \quad (1)$$

where v_1 and v_2 are the volumes of the two solutions taken for a particular mixture. The resistances so calculated for mixtures of NaCl and sodium citrate are given in Column 4 of Table I. They are also shown graphically by Curve C in Fig. 1. It is seen that the theoretical resistances calculated in this way do not differ by more than 1 or 2 per cent from the observed values. The calculated values are all high, however, and the divergence from the observed values increases gradually as the total volume increases.

The theoretical titration curve for CaCl_2 and sodium citrate was also calculated by means of equation (1). The values of the resistances calculated in this way are given in Table II, Column 4; they are also given graphically by Curve D in Fig. 1.

These calculations do not take into consideration the change

produced in the activity coefficient of each salt by the increase in volume. When a given salt solution is mixed with an equal volume of water, the resistance of the dilute solution is not half of the resistance of the original solution but is less than half. This is due to an increase in the activity coefficient or, according to the classical theory, to an increase in the "degree of ionization."

Ruby and Kawai (9) employed several methods of calculating the conductances of mixtures of two solutions from the conduct-

TABLE III.
Conductivity Titration of 5.0 Cc. of 0.005 N CaCl₂ with Water.

Water added.	Total volume.	$R_{\text{obs.}}$	$R_{\text{calc.}}^*$	Resistance of 0.005 N CaCl ₂ corrected for dilution effect.†
(1)	(2)	(3)	(4)	(5)
<i>cc.</i>	<i>cc.</i>	<i>ohms</i>	<i>ohms</i>	<i>ohms</i>
0.0	5.0	1089	1089	1089
1.0	6.0	1282	1307	1068
2.0	7.0	1472	1525	1051
3.0	8.0	1656	1742	1035
4.0	9.0	1838	1980	1021
5.0	10.0	2020	2178	1010
6.0	11.0	2197	2396	999
7.0	12.0	2368	2614	987
8.0	13.0	2543	2831	978

* Calculated, neglecting increased degree of ionization, by means of the equation

$$R_{\text{calc.}} = \frac{v_1 + v_2}{v_1} \times 1089$$

where v_1 = volume of salt solution and v_2 = volume of added water.

$$\dagger \text{ Corrected resistance} = \frac{R_{\text{obs.}}}{R_{\text{calc.}}} \times 1089.$$

ances of the original solutions. For pairs of uni-univalent electrolytes they obtained good agreement between observed results and the calculated values. Renholm (10) applied a modification of the expression of Debye and Hückel (11) to the calculation of conductances of mixtures of KCl and LaCl₃. He obtained almost exact agreement between the values calculated by the modified Debye-Hückel equation and those calculated by the simple rule of mixtures. Our calculated resistances have been obtained according to the simple rule of mixtures.

Since it is uncertain to what extent the theory of complete dissociation of strong electrolytes applies to citrates, we determined the approximate effect of dilution on the change in the activity coefficient of each salt by means of conductivity titrations, performed as follows:

The same volume of 0.005 N CaCl_2 was taken as in the titration with sodium citrate, *i.e.* 5.0 cc. Then water was added, 1.0 cc. at

TABLE IV.

Conductivity Titration of 5.0 Cc. of Water with 0.005 N Sodium Citrate.

Na citrate added.	Total volume.	$R_{\text{obs.}}$	$R_{\text{calc.}}^*$	Resistance of 0.005 N Na citrate corrected for dilution effect.†
(1)	(2)	(3)	(4)	(5)
cc.	cc.	ohms	ohms	ohms
0.0	5.0			
1.0	6.0	4450	5340	742
2.0	7.0	2796	3115	799
3.0	8.0	2188	2373	821
4.0	9.0	1874	2003	833
5.0	10.0	1680	1780	840
6.0	11.0	1547	1632	844
7.0	12.0	1452	1586	847
8.0	13.0	1382	1446	850

Resistance of 0.005 N Na citrate = 899.

* Calculated, neglecting increased degree of ionization, by means of the equation

$$R_{\text{calc.}} = \frac{v_1 + v_2}{v_1} \times 899$$

where v_1 = volume of salt solution and v_2 = 5.0 cc. of water.

$$\dagger \text{ Corrected resistance} = \frac{R_{\text{obs.}}}{R_{\text{calc.}}} \times 899.$$

a time, instead of sodium citrate solution. The solution was stirred and the resistance was measured in the usual way after each addition of water. The results are given in Table III.

To determine the effect of dilution on the resistance of the sodium citrate solution, 5.0 cc. of water were titrated with sodium citrate. Instead of CaCl_2 being used, 5.0 cc. of water were taken initially. To this was added 0.005 N sodium citrate, 1.0 cc. at a time. The resistance was determined after the addition of each aliquot. The results are given in Table IV.

Column 3 in Table III gives the observed resistances and Column 4 gives the resistances calculated without consideration of the increase in activity coefficient with increasing dilution. It is seen that the observed resistances are lower than those calculated in this way and that the differences are greater the more dilute the solution. The calculations in Column 4 are made on the simple assumption that the resistance of a mixture is directly proportional to the total volume and inversely proportional to the volume of added salt solution; *i.e.*, if a given solution has a given resistance, then doubling the volume, by the addition of water, will result in doubling the resistance. The expression used is

$$R_{\text{calc.}} = \frac{v_1 + v_2}{v_1} \times (\text{resistance of the undiluted salt solution}) \quad (2)$$

where v_1 is the volume of salt solution and v_2 is the volume of added water in a given mixture.

Column 5, Table III, gives the resistance of the CaCl_2 solution corrected for the effect of dilution on the activity coefficient. For example, a solution of CaCl_2 with a measured resistance of 1089 ohms, when diluted with an equal volume of water would be expected to have twice the resistance, or 2178 ohms, if the "degree of ionization" did not increase with dilution. When 5 cc. of this CaCl_2 solution are diluted with 5 cc. of water, the measured resistance is 2020 ohms instead of 2178 ohms; *i.e.*, the resistance is less than that calculated by means of equation (2). In order that the resistance calculated by equation (2) shall be equal to that experimentally observed, the resistance of the 0.005 N CaCl_2 solution is corrected by a different factor for each dilution. This is shown in the following equation

$$\text{Corrected resistance} = \frac{R_{\text{obs.}}}{R_{\text{calc.}}} \times 1089^* \quad (3)$$

* The corrected resistance may be obtained without first obtaining $R_{\text{calc.}}$. The expression used is

$$\text{corrected resistance} = \frac{v_1}{v_1 + v_2} \times R_{\text{obs.}}$$

For equation (3), $R_{\text{obs.}}$ is taken from Column 3, Table III, and $R_{\text{calc.}}$ is taken from Column 4. The corrected resistance so obtained is given in Column 5.

An analogous procedure was employed with the data for sodium citrate; the values are given in Table IV.

Theoretical Curves for the Conductivity Titration.—The purpose of all these dilution experiments and calculations was to determine the position and shape the theoretical titration curve would have if no chemical reaction occurred when sodium citrate is added to calcium chloride. In Column 4, Table II, are given the theoretical resistances calculated from equation (1) with the value of 1089 ohms for the CaCl_2 and 899 ohms for the sodium citrate. These theoretical titration values are shown by Curve D in Fig. 1. Inasmuch as the effect of dilution on the increase in the activity coefficient of each salt was neglected, this curve gives not the exact expected resistances, but the probable maximum resistances of mixtures of these two solutions.

Another set of theoretical titration values was calculated also by means of equation (1), but with the values given in Column 5 of Tables III and IV; *i.e.*, with the resistances corrected for dilution. This second set of theoretical titration values is given in Column 5, Table II, and is given graphically by Curve E in Fig. 1. The presence of sodium citrate diminishes somewhat the activity coefficient of the calcium chloride, and the presence of calcium chloride diminishes somewhat the activity coefficient of the sodium citrate; these effects have not been allowed for. Curve E, therefore, does not give the exact position of the curve, but rather the probable minimum position of the curve for mixtures of these two solutions. If no chemical reaction occurred when sodium citrate is added to calcium chloride, the resistances of mixtures of the two solutions would probably lie somewhere between Curves D and E. *The experimental Curve B varies markedly in position from even the highest expected curve; moreover it possesses a maximum, while the theoretical curves have no point of inflection.*

Point to Point Resistances.—It is seen that not only are the experimental resistances greater than the highest values calculated, but that there is also a maximum in the experimental curve. The experimental curve was investigated by means of point to point calculations.

In these point to point calculations, equation (1) was employed, but the values R_1 , v_1 , and v_2 have different significance. The resistance of a given mixture is calculated from the resistance and

volume of the preceding mixture. The calculated resistance of the mixture of 5.0 cc. of CaCl_2 and 1.0 cc. of sodium citrate is the same as is given in Column 4 of Table II; i.e., 1057 ohms. For the next point, with a total volume of 7.0 cc., R_1 is taken as the *measured* resistance of the preceding mixture of 6.0 cc. total volume, while v_1 is 6.0 cc. In all of this series of calculations $R_2 = 899$ ohms and $v_2 = 1$ cc. For this second mixture therefore, the expression

$$R_{\text{calc.}} = \frac{R_1 v_1 + R_2 v_2}{v_1 + v_2}$$

becomes

$$R_{\text{calc.}} = \frac{(6 \times 1220) + (899 \times 1)}{6 + 1} = 1174 \text{ ohms}$$

For the third mixture (total volume = 8 cc.), R_2 is taken as the measured resistance of the second mixture. This gives

$$R_{\text{calc.}} = \frac{(7 \times 1332) + (899 \times 1)}{7 + 1} = 1278 \text{ ohms}$$

The resistances calculated in this way are given in Column 6 of Table II.

The differences between the point to point calculated resistances and the measured resistances are given in Column 7 of Table II. These differences are large for the first additions of sodium citrate to calcium chloride. As more sodium citrate is added, the difference between what is expected and what is observed diminishes; when 5.5 cc. have been added, the difference is less than 1 per cent, *and the observed and calculated resistances agree from this point on to within 1 per cent.*

DISCUSSION.

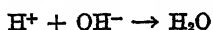
We are interested here in the changes in resistance which occur when these solutions are mixed rather than in the actual resistances themselves. The resistances given in Tables I and II for the three original solutions are not those of pure solutions but of solutions containing some NaCl in addition, since the original solutions were all adjusted to the same pH with either NaOH or

HCl. The position of the titration curves with respect to the Y axis varies with the amount of additional NaCl; the shapes of the curves, however, are not affected, or are only slightly affected, by the presence of NaCl.

In the control titration of NaCl with sodium citrate, the observed results agree well with the theoretical. The resistance of the citrate solution is greater than that of the NaCl solution. Mixtures have resistances as expected; *i.e.*, intermediate between those of the two original solutions. Moreover, the resistance of any given mixture agrees within about 1 per cent with that calculated from equation (1). The greater the volume of added sodium citrate, the greater is the calculated resistance, and also that of the observed resistance—there is no maximum or minimum in either the theoretical curve, Curve C, or the experimental curve, Curve A.

For mixtures of calcium chloride and sodium citrate, a similar theoretical curve, Curve D, was obtained. In this theoretical curve, the resistances of mixtures decrease progressively with increasing proportions of sodium citrate solution. The experimental curve, Curve B, however, is strikingly different from the theoretical—addition of sodium citrate causes the resistance to increase instead of decrease. The resistance continues to increase to a maximum; only after about an equal volume of the citrate solution has been added, does the resistance decrease as expected. For a mixture containing 5.0 cc. of each solution, the calculated resistance from Curve D is 994 ohms while the observed resistance is 1470 ohms; *i.e.*, the observed resistance is about 50 per cent higher than the theoretical. If we employ the other theoretical curve, Curve E, the difference is still more marked and is in the neighborhood of 60 per cent.

The solutions were adjusted to the same pH before the titration was begun; at the end of the titration, the pH of the mixture was still 7.4. The peculiar observed increase in resistance to a maximum cannot, therefore, be attributed to withdrawal of hydrogen and hydroxyl ions from the solution because of the reaction



There seems to be but one possible cause of this observed increase in resistance; namely, a decrease in the number of ions. Mixing

sodium ions and citrate ions with calcium ions and chloride ions, all at the same pH, apparently is followed by a decrease in the total number of ions. This ionic reaction cannot be due to the effect of the sodium citrate on the chloride since normal results are obtained when the sodium citrate solution is added to one of sodium chloride. The reaction must therefore involve the calcium ions.

Calcium ions appear therefore to be removed from solution by addition of sodium citrate. The nature of the calcium citrate complex which is so formed cannot be determined without further study. It is possible that calcium ion unites with citrate ion to form a neutral compound or a complex negatively charged ion, or a complex positively charged ion. Apparently all of the calcium can be bound in this way as evidenced by the point to point calculated resistances, given in Table II. The observed resistances for the first additions of sodium citrate are much higher than the resistances calculated in this way; as more citrate is added, the discrepancies diminish. After an equal volume of citrate has been added, the observed and calculated point to point resistances agree to within 1 per cent. (See also Fig. 1.)

This means that when sodium citrate is first added to a solution of calcium ions, an ionic reaction occurs which results in a decrease in the number of ions. With further addition of sodium citrate, fewer and fewer ions are removed from solution. When the reaction is complete, further addition of sodium citrate does not cause any further diminution in the number of ions; the resistance from this point on therefore decreases in agreement with the calculated values.

SUMMARY.

1. Conductivity titration of sodium chloride with sodium citrate at 25° gives results which are normal, and which are in good agreement with calculated values.

2. Similar conductivity titrations of calcium chloride with sodium citrate give abnormal results. The observed resistance, instead of decreasing, increases to a maximum; after the maximum is passed it decreases in agreement with theory.

3. These results are direct evidence for the binding of calcium ions by sodium citrate in some kind of soluble complex.

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THE ELECTRICAL TRANSFERENCE OF CALCIUM IN BLOOD SERUM PROTEIN SOLUTIONS.

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INTRODUCTION.

In a paper by Updegraff, Greenberg, and Clark (1) there was stated "Rona and Takahashi [(2)], in 1911 and 1913, suggested that the non-diffusible calcium was bound to the proteins of the serum and this was tacitly accepted by all later investigators until the publication by Cameron and Moorhouse [(3)], in 1925, of their paper 'Tetany of Parathyroid Deficiency.' In this paper Cameron and Moorhouse rejected the above hypothesis and proposed instead that the non-diffusible calcium is bound to some organic substance which may be the parathyroid hormone."

Since then experimental work to demonstrate that the non-diffusible calcium is chiefly held in the form of a Ca-protein complex ion has been published by Marrack and Thacker (4), Loeb (5), and Loeb and Nichols (6). Of these studies the most complete is that of Loeb and Nichols. By membrane distribution experiments, these authors have shown that the amount of calcium inside of sacs containing serum or serum proteins was from 30 to 50 per cent higher than would be expected from the distribution ratios of chloride ion in dialysate and serum.

In the present work the attempt is made to demonstrate the existence of complex Ca-protein ions by the electrical transference method.

EXPERIMENTAL.

The experimental work was carried out on electrodialyzed beef serum. To decrease the time of centrifuging and obtain

* Aided by a grant from the Research Board of the University.

a complete separation of the serum, the blood was made somewhat hypertonic by the addition of a 0.1 volume of 1.4 per cent sodium chloride solution. About 1 liter of such a serum was placed in the middle compartment of a dialyzer and dialyzed electrolyte-free, until the specific conductivity fell to from 1 to 2×10^{-5} mhos. The dialyzer was made by sawing a 6 volt, rubber, automobile battery case into three sections. The membranes were a parchment cathode and chrome gelatin anode as described by Freundlich and Farmer-Loeb (7). The pH of the electrodialed serum was found to be between 5.6 and 5.7. The dialyzed serum was kept in a refrigerator under toluene and samples were withdrawn for use as needed.

The analytical methods employed were the following.

The protein was determined by drying 10 gm. portions of solution in porcelain crucibles at 105° and weighing. From this weight was subtracted the weight of the cation in the solution. The titratable base in the solutions, which in these experiments represents the sum of the sodium and calcium present, was determined by electrometric titration to pH 5.6 with the hydrogen 0.1 N calomel electrode, 0.025 N HCl being used. Schmidt and Hoagland's tables (8) were used to obtain the pH values from the E. M. F. readings. The calcium itself was determined by the Van Slyke and Sendroy (9) gasometric method.¹ The amount of sodium present was obtained by difference.

The transference experiments were carried out in duplicate, at 25° , in the same kind of cells and with the same technique as were previously used in casein transport number studies (10). In these solutions, unlike that of casein, there was no adhering deposit of protein to the platinum anode. This made it necessary to calculate the transport numbers of the proteins of the solutions in a different manner than heretofore. The method employed was to assume the equivalent weight of the protein to be the reciprocal of the equivalents of base added per gm. of protein. The amount of protein gained by the anode, calculated in equivalents, divided by the equivalents of current passed through the solution gives the transference number. Since there are a number of proteins in the solution, no significance can be placed on the

¹ I am indebted to Mr. H. E. Ballard for carrying out the calcium analyses.

absolute value of the transport numbers calculated in this way. However, it seems that the calculated transport numbers should bear some constant relationship to the true transport numbers of the protein.

DISCUSSION.

In an attempt to get a picture of the carrying of the current in its simplest possible terms, the first measurements were made on

TABLE I.
Transport of Electrodialyzed Serum in a Single Alkali Solution.

Serum sample.	Serum proteins.	Alkali per gm. of protein.	pH	T_{serum}^-	T_{cation}^+
Alkali, NaOH.					
S ₂	<i>per cent</i>	<i>m.-eq.</i>			
	2.0	0.28			0.60
	2.0	0.28			0.57
S ₃	1.2	0.24	7.9	0.25	
	1.2	0.24	7.9	0.25	
	3.5	0.23	7.9	0.18	0.70
S ₄	3.5	0.23	7.9	0.19	0.61
	3.3	0.25	8.2	0.23	
	3.3	0.25	8.2	0.21	
Average.....				0.22	0.62
Alkali, Ca(OH) ₂ .					
S ₁	3.2	0.26	8.2	0.32	
	3.2	0.26	8.2	0.32	
	3.2	0.27	8.8	0.27	0.48
S ₃	3.2	0.27	8.8	0.27	0.55
	4.4	0.22	8.0	0.21	0.75
	4.4	0.22	8.0	0.20	(0.95)?
Average.....				0.27	0.60

solutions to which either NaOH or Ca(OH)₂ only had been added. The results of these measurements are given in Table I. From Table I it is to be seen that sodium and calcium ions carry about the same fraction of the current. From the mobilities of the sodium and calcium ions, which are 50 and 60 mhos respectively at 25°, it would be expected that the transference numbers of

these ions should be in that ratio, providing there are no complicating factors. In this instance, due to the formation of a complex ion of protein and calcium, some calcium will be carried in the opposite direction to its normal movement. As a result the

TABLE II.

Electrical Transference of Electrodialyzed Serum in Mixed Solutions of Sodium and Calcium Hydroxide.

Serum sample.	Base added.			pH	Ratio of Ca(OH)_2 * Titratable base	T serum proteins.	T titratable base.*	$T \frac{1}{2} \text{Ca}^{++}$.		Loss of Ca from anode.	
	Serum proteins.	NaOH per gm. protein.	Ca(OH)_2 per gm. protein.					Obtained.	Calculated.	Found.	Calculated.
	(1)	(2)	(3)								
	per cent	m.-eq.	m.-eq.		per cent					mg.	mg.
S_3	4.00	0.195	0.055	8.0	22	0.23		(-0.08)	0.15	(-1.15)	2.2
	4.00	0.195	0.055	8.0	22	0.24		(-0.08)	0.15	(-0.65)	1.3
S_4	3.45	0.226	0.07	9.3	24	0.31	0.52	(-0.09)	0.16	(-0.65)	1.2
	3.45	0.226	0.07	9.3	24	0.32	0.51	(-0.11)	0.16	(-0.50)	0.8
	3.49	0.135	0.128	8.8	49	0.29	0.49	(-0.02)	0.33	(-0.05)	2.0
	3.49	0.135	0.128	8.8	49	0.30	0.55	0.0	0.33	0.0	1.3
	3.43	0.204	0.048	8.7	19	0.28		0.0	0.13	0.0	1.1
	3.43	0.204	0.048	8.7	19	0.27		0.01	0.13	(-0.1)	0.7
	3.67	0.174	0.09	8.6	34	0.31	0.62	0.0	0.23	0.0	1.6
	3.67	0.174	0.09	8.6	34	0.31	0.49	0.03	0.23	0.3	1.0
S_5	4.22	0.168	0.079	8.3	32	0.27		0.0	0.21	0.0	1.5
	4.22	0.168	0.079	8.3	32	0.25		(-0.02)	0.21	(-0.1)	1.0
	3.70	0.199	0.088	8.4	30	0.29		0.03	0.20	0.15	1.9
	3.70	0.199	0.088	8.4	30	0.30		0.05	0.20	0.15	1.2
	4.26	0.184	0.048		21	0.23	0.63	0.04	0.14	0.3	1.1
	4.26	0.184	0.048		21	0.21	0.66	0.04	0.14	0.2	0.8
Average.....						0.28	0.56				

* Titratable base represents the sum of sodium and calcium.

calculated transport numbers will be lower depending upon the amount of the complex ion, and may even become negative in sign. The amount of complex formation that might be expected, with the non-diffusible calcium (1) as a measure, is not over 0.04 milli-equivalents of calcium per gm. of protein. Since this is

less than 20 per cent of the total calcium in the above solutions, it is not surprising that it does not show to any great degree in the transference results of the solutions containing only calcium hydroxide.

To attempt to reduce the concentration of calcium so that if complex ions were present they would have a significant effect on the calcium transference, experiments were next carried out with solutions of electrodyalyzed serum containing mixtures of sodium and calcium hydroxide. The results of these experiments are given in Table II. The column headings are self-explanatory. In Columns 8 and 9 are given the transport numbers of calcium as actually determined by analysis, and as calculated from the concentration of calcium in the solution and its mobility, no complex ion formation being assumed. For the calculation of the expected transport number of the calcium, the average figure of the titratable base transport, 0.56, was taken as a basis. This figure multiplied by the ratio of calcium to titratable base in the solution (Table II, Column 5) and corrected for the difference in mobility by multiplying by $\frac{6.0}{5.0}$ gives the expected calcium transport number, with the assumption of no complex ion formation. It is to be seen that the transport numbers found in all cases diverge widely from the calculated numbers and in some cases are even opposite in sign. To show that errors in analysis cannot account for the low transference numbers obtained, there are given in the last two columns the found and calculated loss of calcium from the anode compartment for the experiments, in mg. of calcium. The maximum possible error of analysis would be about 0.2 to 0.3 mg. The difference between the found and calculated loss is usually many times this amount.

The results, then, indicate that there are complex ions of calcium and the serum proteins in their respective solutions. This is in harmony with the findings of Greenberg and Schmidt (11) and of Kirk and Schmidt (12) on solutions of casein in the alkali earth hydroxides. The amount of complex ion formation with the serum proteins is much less, however, than with casein.

SUMMARY.

By the electrical transference method, the presence of complex Ca-protein ions was demonstrated in solutions of electrodyalyzed

blood serum to which mixtures of sodium and calcium hydroxide were added.

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STUDIES OF GAS AND ELECTROLYTE EQUILIBRIA IN BLOOD.

XII. THE VALUE OF pK' IN THE HENDERSON-HASSELBALCH EQUATION FOR BLOOD SERUM.

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Hasselbalch in 1917 proposed the now widely applied calculation of the blood pH from the CO_2 content and tension by means of Henderson's (1909) equation in the logarithmic form

$$(1) \quad \text{pH} = \text{p}K' + \log \frac{[\text{BHCO}_3]}{[\text{H}_2\text{CO}_3]}$$

In this equation pK' is the negative logarithm of the apparent dissociation constant, K' , of the more acid hydrogen atom of H_2CO_3 . K' is a collective constant. It includes not only the first acid dissociation constant of the acid, H_2CO_3 , but also the equilibrium constant of the reaction, $\text{CO}_2 + \text{H}_2\text{O} = \text{H}_2\text{CO}_3$, the activity coefficient of the HCO_3 anion, and the dissociation coefficient of BHCO_3 , in case its dissociation is not complete.

The value of pK' was determined by Hasselbalch (1917) for solutions containing NaHCO_3 and H_2CO_3 in the range of concentrations found in the blood, and was found to be about 6.1 at 38° , when both H_2CO_3 and BHCO_3 were expressed as molal concentrations. Warburg (1922) studied the value of pK' further in salt solutions; and Hastings and Sendroy (1925) showed that pK' increased in a linear manner with the square root of the ionic strength of the salt solutions studied, in accordance with the theory of Debye and Hückel. Van Slyke, Hastings, Murray, and Sendroy (1925) also showed that the pK' value for serum

approximates that of a salt-bicarbonate solution of similar ionic strength.

In whole blood Hasselbalch (1917) found that pH values calculated with the same pK' constant that he had determined in bicarbonate solutions approximated the pH values found with the hydrogen electrode. Parsons (1917) demonstrated that the pH determined electrometrically in whole blood is really that of the serum, the suspended corpuscles having no direct influence on the results. Since the BHCO_3 content per unit volume of the corpuscles is much less than that of the serum, and to a degree variable with the pH, any pK' value for whole blood must contain an added, variable factor, to correct for the unequal BHCO_3 distribution. The effect of hemoglobin content, degree of oxygenation, and serum pH on this factor has been studied theoretically and experimentally by Warburg (1922), by Van Slyke, Wu, and McLean (1923), and by Peters, Bulger, and Eisenman (1923). The number of factors affecting the value of pK' in whole blood indicates the desirability, in estimating serum pH by Hasselbalch's method, of making the CO_2 determinations when possible on serum rather than whole blood.

The data in the literature from which the pK' for blood serum can be calculated have been reviewed by Warburg (1922), and more recently by Cullen, Keeler, and Robinson (1925). There still exists a considerable divergence in results concerning the figure in the second place decimal of the pK' for serum, which we shall designate as pK_s' . The purpose of the present paper is to add to the available data a number of determinations on human sera, on which not many values have been reported, and to summarize for comparison the results obtained by other investigators with the more modern methods.

The value of pK' is calculated from experimentally determined values of pH and the molal concentrations, $[\text{BHCO}_3]$, $[\text{H}_2\text{CO}_3]$, according to the rearranged Hasselbalch equation,

$$(2) \quad \text{pK}' = \text{pH} + \log [\text{H}_2\text{CO}_3] - \log [\text{BHCO}_3]$$

In calculating the value of pK' the solubility coefficient of CO_2 plays an important part not indicated by the above equation. The value of $[\text{H}_2\text{CO}_3]$ has in all published determinations been fixed by saturating the solution with CO_2 under a known tension,

and estimating the H_2CO_3 as the physically dissolved CO_2 , according to Henry's law, expressed in the equation

$$(3) \quad [\text{H}_2\text{CO}_3] \text{ in cc. CO}_2 \text{ per 100 cc. solution} = \alpha \times \frac{p}{760}$$

or

$$(4) \quad [\text{H}_2\text{CO}_3] \text{ in mm per liter solution} = \frac{\alpha p}{22.26 \times 0.76} = 0.0591 \alpha p$$

where p is the CO_2 pressure in mm., and α is Bunsen's solubility coefficient, representing the cc. of CO_2 , measured at 0° , 760 mm., that are dissolved by 1 cc. of solution when under 760 mm. pressure of the gas. As a rule, the value of $[\text{BHCO}_3]$ has been determined by finding the total CO_2 content of the solution, and subtracting therefrom the H_2CO_3 . The equation expressing the calculation of pK' from the directly determined values therefore is:

$$(5) \quad \text{pK}' = \text{pH} + \log 0.0591 + \log \alpha + \log p - \log ([\text{CO}_2] - 0.0591 \alpha p)$$

where $[\text{CO}_2]$ represents total CO_2 content of the solution in millimols per liter.

It is evident from the third term of the right hand member that the value of pK' will vary in direct proportion to the logarithm of the value used for the solubility coefficient, α . The latter also influences the last term, but with the relative proportions of total and dissolved CO_2 found in serum the effect of α on pK' through its influence on the last term is not significant.

In a recent paper from this laboratory (Van Slyke, Sendroy, Hastings, and Neill (1928)), the value of α for serum has been directly determined for the first time, and found to be 0.510. It is used for the calculation of pK' from the data given below, and for recalculation of similar data of other authors in the literature.

Methods.

Samples of human serum were equilibrated with mixtures of air and CO_2 in double chambered tonometers according to the "First Saturation Method" of Austin *et al.* (1922).

The total CO_2 content of the saturated serum was determined in the manometric apparatus of Van Slyke and Neill (1924) the

TABLE I.
Determinations of pK' Value of Human Sera.

Subject and serum No.	H ₂ O content.	Total CO ₂ content.		CO ₂ tension.	H ₂ CO ₃ content.*		BHCO ₃ content.		P corrected to 760 mm. H ₂ pressure.	pH	pK'	
		mm per l.	mm per kg. H ₂ O		mm per l.	mm per kg. H ₂ O	mm per l.	mm per kg. H ₂ O			From data in mm per liter.	From data in mm per kg. H ₂ O.
Normal. H-1..... H-2.....	0.923	27.73	30.04	34.8	1.048	1.138	26.08	28.90	998.4	7.510	6.105	6.105
	0.923	30.52	33.07	58.9	1.773	1.927	28.75	31.14	986.0	7.308	6.098	6.100
Normal. O-1..... O-2.....	0.926	23.92	25.83	36.1	1.087	1.181	22.83	24.05	994.1	7.441	6.119	6.122
	0.926	26.41	28.52	60.4	1.818	1.976	24.59	26.54	980.9	7.225	6.094	6.097
Normal. W-1..... W-2.....	0.930	28.94	31.12	36.2	1.090	1.184	27.85	29.94	998.1	7.503	6.096	6.100
	0.930	31.38	33.74	61.4	1.848	2.008	29.53	31.73	986.3	7.313	6.110	6.114
Rheumatic. M-1..... M-2.....	0.927	27.35	29.50	36.1	1.087	1.181	26.26	28.32	996.9	7.486	6.103	6.106
	0.927	29.69	32.03	61.1	1.840	1.998	27.85	30.03	984.5	7.285	6.105	6.108
Rheumatic. H-1..... H-2.....	0.918	29.64	32.29	36.4	1.096	1.190	28.54	31.10	998.7	7.515	6.097	6.098
	0.918	32.09	34.96	61.2	1.843	2.001	30.25	32.96	986.5	7.318	6.103	6.102
Nephritic. G-1..... G-2.....	0.927	31.08	33.53	36.3	1.093	1.187	29.99	32.34	701.1	7.553	6.115	6.118
	0.927	33.88	36.55	59.7	1.800	1.952	32.08	34.60	689.9	7.371	6.120	6.122

results being calculated by the revised factors of Van Slyke and Sendroy (1927).

The CO_2 content of the gas phase in the tonometer at the end of equilibration was determined by Y. Henderson's modification (1918) of the Haldane apparatus for air analysis.

The water contents of the sera were determined by drying 2 cc. samples to constant weight at 110° .

The pH was determined at 38° with the hydrogen electrode. The rocking electrode chamber devised by Clark (1915) was employed, with the modification introduced by Cullen (1922) to permit the insertion of a thermometer. The system used and the standardization with 0.1 N HCl have been described on pages 708 and 709 of Paper VIII of this series (Van Slyke, Hastings, Murray, and Sendroy, (1925)). The reasons for the choice of 0.1 N HCl, with an assumed pH of 1.08 as the solution for determining E_0 in standardization of the cells, have been adequately discussed by Cullen, Keeler, and Robinson (1925).

Calculations.

The CO_2 tension, p , has been calculated from the CO_2 content of the air, with which the serum was equilibrated at atmospheric pressure, by the equation

$$(6) \quad p = \frac{(\text{per cent } \text{CO}_2 \text{ in gas})}{100} \times (B - W)$$

H_2CO_3 concentrations were calculated by Equation 4, where B is the barometric pressure and W is the vapor tension of water.

BHCO_3 concentrations were calculated by subtracting $[\text{H}_2\text{CO}_3]$ from total CO_2 content of the serum. In no case was the pH sufficiently high to permit the presence of a significant amount of B_2CO_3 .

pH was calculated from the observed electromotive force, E , as

$$(7) \quad \text{pH} = \frac{E - E_0}{0.06169}$$

The value of E_0 was determined from the E.M.F. observed with 0.1 N HCl in the hydrogen electrode vessel:

$$(8) \quad E_0 = \text{E.M.F.} - 1.08 \times 0.06169$$

the pH of 0.1 N HCl being assumed to be 1.08.

pK' was calculated by Equation 2 the values of $[H_2CO_3]$ and $[BHC O_3]$ having been estimated as above.

The results of our determinations are summarized in Table I.

Recalculation of pK_s' Values in the Literature.

In the literature of the past 6 years are several papers presenting carefully obtained data, from which the pK' for serum at 38° either has been or may be calculated. In each case one or more of the constants involved in the calculation of results has been slightly or appreciably different from the constants employed in the present paper. In order to make the different pK_s' values comparable, therefore, we have recalculated them from the experimental data with the same factors, standards, and constants used above.

Warburg (1922).—At the end of Table XIII in Warburg's paper are data on twenty sera, of horse, ox, and man, at 38° . The values given are the mm. of CO_2 tension, the $[BHC O_3]$, and the pH determined with Bjerrum's standard, which, as pointed out on page 167 of Warburg's paper, gives pH values 0.048 higher than the Sørensen conductivity standard. The differences in the methods of calculating pH from observed electromotive force, E , depend upon the value taken for E_0 in the Sørensen (1912) equation.

$$pH = \frac{E - E_0}{0.0577 + 0.0002(t^\circ - 18^\circ)}$$

In our determinations, in which E_0 is determined with 0.1 N HCl, assumed to have pH of 1.08, the results approximate closely those of the Sørensen standard. We have therefore subtracted 0.048 from Warburg's pH values, in order to bring them to the same standard as ours. Exactly how precise this correction is we are not certain, but it appears to be within 0.01 pH. With the values of pH thus corrected, we have calculated pK_s' from Warburg's data by means of Equations 2 and 3, given above.¹

¹ Warburg (1922) states that the difference between Bjerrum's and Sørensen's E_0 values for the 0.1 N calomel cell is 2.9 millivolts at room or body temperature. This means that pH calculated from Bjerrum's value would be 0.048 pH higher than that calculated from Sørensen's value. In a footnote on page 166 (1922) he states that the Bjerrum value at 38° is 331.3 millivolts. Sørensen's value for the 0.1 N calomel cell is 336.1 milli-

Cullen (1922).—Cullen published, from eleven analyses of horse plasma and six of horse serum, values for CO₂ contents, CO₂ tensions, and pH, at 38°. For standardizing his hydrogen electrodes Cullen used 0.1 N HCl, but assumed its pH to be 1.09, instead of 1.08. We have accordingly, for the present calculations, subtracted 0.01 from each of his pH values.

TABLE II.
pK' Values Determined in Plasma or Serum by Different Authors.

Authors.	Material.	No. of determinations.	pK _s ' with $\alpha = 0.510$.			pK _s ' with $\alpha = 0.541$ (average).
			Minimum.	Maximum.	Average.	
Warburg (1922)....	Horse and ox serum.	20	6.07	6.13	6.10	6.126
Cullen (1922).....	Horse oxalate plasma.	11	6.086*	6.123	6.110*	6.136
".....	Horse serum.	6	6.105*	6.119	6.113*	6.139
Cullen, Keeler, and Robinson (1925)...	Human "	15	6.043	6.095	6.064	6.090
Van Slyke, Hastings, Murray, and Sendroy (1925)...	Horse "	13	6.09	6.15*	6.13*	6.156
Present writers....	Human "	16	6.094	6.134	6.105	6.131
Mean.....					6.104	6.130

* Excluding one determination, deviating rather widely from the rest of the series.

His determinations of the CO₂ content, in mm per liter, were carried out according to Van Slyke and Neill (1924). With Van Slyke and Sendroy's (1927) more exact factors now used in calculating the results of the Van Slyke-Neill analyses, the CO₂ concentrations in mm are 1.009 times as great as those calculated from the same analyses by the original factors given by Van Slyke and

volts at 38°. This would mean a difference of 4.8 millivolts corresponding to 0.078 pH. Since no original E.M.F. data are given, it is impossible to determine what value for the 0.1 N calomel cell he used in calculating pH, at 38°. In the absence of further information we have assumed that the value of 333.2 millivolts was used.

Neill, and used by Cullen. We have accordingly multiplied the CO_2 values of Cullen by 1.009 in order to make them comparable with our present data. The change effected by this correction is only to lower pK_s' by 0.004.

Cullen, Keeler, and Robinson (1925).—These authors have published data from analyses of fifteen human sera. They have used the same pH standard employed by ourselves. Their serum CO_2 contents were calculated by the original factors of Van Slyke and Neill, and we have corrected them with the factor 1.009, as above outlined.

In the calculations of $[\text{H}_2\text{CO}_3]$ from CO_2 tension Cullen, Keeler, and Robinson used the Bohr (1905) α of 0.541. The change to the 0.510 value, directly determined by Van Slyke, Sendroy, Hastings, and Neill (1928), lowers by 0.026 the pK_s' values calculated by Equation 5. The total effect of the two changes, required in order to calculate the data of Cullen, Keeler, and Robinson on the same basis of those of the present paper, is to lower the pK_s' values calculated from their determinations by 0.030 below the values computed by them from the same determinations.

Van Slyke, Hastings, Murray, and Sendroy (1925).—The pH standard was the same used in the present paper. The serum CO_2 contents given by these authors we have multiplied by 1.009 to change them to the values calculated by the new factors of Van Slyke and Sendroy (1927). The pK_s' values are then recalculated by means of Equation 5.

The pK_s' values calculated from the data of the above authors, together with our own, are summarized in Table II.

CONCLUSIONS.

The variations, due to causes which cannot be ascertained at present, are such that one does not appear justified in expressing the value of pK_s' to more than two decimal places. Consideration of the available data indicates that, when the solubility coefficient of CO_2 in serum is taken as 0.510, and the pH of 0.1 N HCl is taken as 1.08 in standardizing the electrometric pH determinations, the value of pK' for serum at 38° is approximately 6.10.

The pK_s' calculated from the same data with the Bohr α , 0.541, used by most previous authors, would be 6.13.

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STUDIES OF GAS AND ELECTROLYTE EQUILIBRIA IN BLOOD.

XIII. THE DISTRIBUTION OF CHLORIDE AND BICARBONATE IN THE BLOOD OF NORMAL AND PATHOLOGICAL HUMAN SUBJECTS.

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In 1923, Van Slyke, Wu, and McLean (1) published the results of a study of the factors responsible for the unequal distribution of hydrogen, chloride, and bicarbonate ions between erythrocytes and blood serum. This study was made upon blood brought into equilibrium, *in vitro*, with definite tensions of carbon dioxide and oxygen. Under these conditions it was found that the quantitative distribution of the diffusible ions could be approximately predicted by equations which were developed from the assumptions that osmotic pressure is equal in cells and serum, and that the distribution of diffusible ions between cells and serum is influenced by the non-diffusible ions according to the Gibbs-Donnan law. A later and more accurate experimental examination of the problem (2) emphasized the necessity of taking into account the factors which reduce the activity of ions in cells and serum and verified the fundamental correctness of the original postulates and equations. That the non-diffusible base bound by hemoglobin in the cells is the most important single constituent of blood in determining the distribution of chloride, bicarbonate, and hydrogen ions, seems established from the fact that any change, such as oxygenation or shift in reactions, which alters the amount of base bound by hemoglobin, brings about a predictable change in ionic distribution.

After the factors concerned with ionic distribution in horse blood brought artificially into equilibrium with O_2 and CO_2 had been

investigated, it seemed pertinent to study ionic distribution in blood freshly drawn from the human body.

This has been done with blood from normal individuals and from a series of hospital patients suffering from a variety of acute and chronic pathological conditions. As far as possible patients were chosen in whom there appeared to be the greatest probability of abnormal ionic distributions; *i.e.*, cardiac and pneumonia patients, in whom oxygenation may be deficient; nephritic patients, who may be anemic, have low plasma proteins, nitrogen retention, and acidosis; febrile patients with pneumonia and acute arthritis.

The following determinations have been made when adequate material was available: on the serum, electrometric pH, total CO_2 , total base, chloride, water, and protein; on the cells, total CO_2 , chloride, and water; on the whole blood, total oxygen content and capacity.

Our series consisted of nine observations on eight normal and thirty observations on twenty-nine pathological individuals. The latter were distributed as follows:

Disease.	No. of observations.
Pneumonia.....	9
Nephritis.....	13
Arthritis.....	5
Cardiac disease.....	3

Technique.

About 40 cc. of venous blood were drawn without stasis and defibrinated under oil. A sample of whole blood, about 6 cc. in amount, was transferred to a separate tube immediately for determination of its oxygen content and capacity. 0.1 cc. of 0.1 N KCN was added to the remainder for each 10 cc. of blood to inhibit respiration. The oil on the blood was replaced with paraffin, and the blood was centrifuged for 45 minutes at high speed. The bulk of the serum was then transferred to small sampling tubes over mercury. The last visible portion of the serum was removed by suction cautiously and slowly applied. The cells were then similarly transferred to sampling tubes over mercury, where they were kept for analysis. The CO_2 determinations were done immediately.

pH of serum was determined electrometrically at 38° in a Clark cell (3) as modified by Cullen (4). All values have been calculated

on the assumption that the pH of 0.1 N HCl is 1.080. No correction was attempted for diffusion potentials. Details of the method and of the calculation have been described in Paper VIII of this series (2).

Carbon dioxide and oxygen determinations were performed with the Van Slyke and Neill (5) manometric blood gas apparatus. The cell samples for CO₂ analysis were measured by weight. The sample was drawn into a 1 cc. stop-cock pipette, which was weighed. The sample of approximately 1 cc. was then delivered into the chamber of the Van Slyke-Neill apparatus, together with 6 cc. of N/60 lactic acid, which had previously been rendered CO₂-free by extraction in the apparatus. The pipette was then weighed again. The CO₂ values were calculated by the revised factors of Van Slyke and Sendroy (6).

Chlorides were determined in weighed samples of serum and cells by the method of Van Slyke (7). In the procedure the serum or cells are mixed in small Erlenmeyer flasks with 3 volumes of concentrated nitric acid containing a known amount of silver nitrate, and heated on a steam bath until the organic matter is destroyed and the chloride is precipitated as AgCl. The excess silver in solution is then titrated with sulfocyanate. A test of this method on sera and cells of various animal species, carried on in the laboratory of Professor D. Wright Wilson in Philadelphia and of the authors in New York (unpublished) has shown that in an occasional abnormal human serum, for a reason not yet identified, this method yields results 1 or 2 per cent too low. The method revealed no errors in cell analyses. In sera which gave low results, correct ones were obtained when the silver nitrate in water solution was mixed with the serum before nitric acid was added. This improvement, due to Professor Wilson, had not been introduced at the time the present work was done. The possible error of the original method is, however, not sufficient to be of significance in interpretation of our results.

Total base determinations were made by the modification of Fiske's benzidine sulfate method described on page 671 of the paper by Van Slyke, Hiller, and Berthelsen (8). Their gasometric micro method was not yet perfected at the time of the work here reported.

Serum proteins were estimated by gasometric micro-Kjeldahl determinations (9).

Values of water per gram or cc. were obtained by drying a known weight or volume to constant weight at 110° .

All the above determinations were done in duplicate, with good agreement. Consequently we believe that significant variations due to analytical error are excluded.

Calculations.

CO_2 tension of the blood was calculated from the total CO_2 content of the serum and the electrometrically determined pH_s by Formula 5 of Table IV of Paper I of this series (10).

$$p = \frac{[\text{CO}_2]}{0.0591 \alpha^0 (10^{\text{pH} - \text{pK}'} + 1)}$$

The value for pK' , from the accompanying paper (11) is taken as 6.10. The value of α^0 , which indicates the cc. of CO_2 dissolved per gram of water in serum under 760 mm. CO_2 tension, is taken as 0.553 (12). Hence the equation becomes

$$p = \frac{[\text{CO}_2]}{0.0327 (10^{\text{pH}_s - 6.10} + 1)}$$

mm $[\text{H}_2\text{CO}_3]_s$ per kilo of water in serum was calculated, from the above value of α^0 , as

$$[\text{H}_2\text{CO}_3]_s = 0.0327 p$$

mm $[\text{H}_2\text{CO}_3]_c$ per kilo of water in cells was calculated, from the value $\alpha^0 = 0.60$, found for cells (12), as

$$[\text{H}_2\text{CO}_3]_c = 0.0354 p$$

Bicarbonate was estimated as the difference between total CO_2 and H_2CO_3 .

$$[\text{BHCO}_3] = [\text{CO}_2] - [\text{H}_2\text{CO}_3]$$

The analyses were all performed on the partially oxygenated venous blood. In order to estimate the $[\text{Cl}]_c : [\text{Cl}]_s$ and $[\text{BHCO}_3]_c : [\text{BHCO}_3]_s$ ratios for a standard condition, for comparison of the results from different bloods, we have calculated in each case what the ratio would be if the blood were completely oxygenated. For this purpose we have used the empirical equation

$$c = 0.1 (\text{pH}_s - 6.6) \frac{\text{reduced Hb}}{\text{total Hb}}$$

where c is the amount by which the determined ratios must be decreased to give the ratios of completely oxygenated blood. This empirical equation was deduced from results obtained with oxygenated and reduced horse blood in Paper VIII of this series (2). This c correction, although probably not strictly accurate for human blood, is in all cases so small that the error involved from basing it on horse blood data is negligible.

DISCUSSION.

The outstanding feature of the r_{Cl} and r_{HCO_3} values for human blood diagrammed in Fig. 1 is that in both normal and pathological cases the values at any given pH, average definitely higher than those found for horse blood (2). To seek the factor which may cause this difference we inspect Equation 10 of Van Slyke, Wu, and McLean, in which they expressed a first approximation of the main factors affecting r , the ratio of the active concentration of Cl or HCO_3 anions in cells to the active concentration in serum.

$$r = 1 - \frac{[\text{BP}]_c + [\text{Hb}]_c - [\text{BP}]_s}{2 ([\text{B}]_c - [\text{BP}]_c)}$$

In the fraction on the right, which determines by how much r falls below unity, the two most important values at physiological pH ranges are $[\text{BP}]_c$, the base bound by cell colloids, and $[\text{B}]_c$, the total serum base. The latter is practically the same in horse blood as in human, about 170 to 175 milli-equivalents per kilo of serum water, or 156 to 160 milli-equivalents per liter of serum. But the $[\text{BP}]_c$ values appear to be appreciably different in the two species. Comparison of the data of Van Slyke, Wu, and McLean (1) on horse cell contents with those of Adair (13) on human cell contents indicates that the colloids of the latter bind less base at a given pH. In both cases (1, 13) the determinations were made of the base-binding power of the entire non-dialyzable constituents of the cells, which include, beside hemoglobin, other constituents, perhaps phosphatides, which bind less, but important, amounts of base. Thus Fig. 16 of our Paper VIII (2) indicates that at pH 7.4 recrystallized reduced horse hemoglobin binds per mol 1.7 equivalents of alkali, while Fig. 10 of Van Slyke, Wu, and McLean (1) indicates that at the same pH the dialyzed total cell contents bind 2.2 equivalents of alkali per mol of reduced hemoglobin.

The non-hemoglobin colloids of horse cells therefore appear to bind at pH 7.4 about 0.5 equivalent of base per mol of Hb. All such non-diffusible acids would, according to Van Slyke, Wu, and McLean, act alike in affecting the electrolyte distribution; hence all the base bound by non-diffusible cell constituents is represented by one symbol $[\text{BP}]_c$.

An estimate of the quantitative effect on the Cl and HCO_3 distribution ratios at normal blood pH, which would be caused by the difference between $[\text{BP}]_c$ found in horse cell contents (1) and in human cell contents (13) may be made as follows.

At pH 7.2, which is about the cell pH of normal blood (1, 2) Fig. 10 of Van Slyke, Wu, and McLean (1) indicates that the non-diffusible constituents of oxygenated horse cells bind 2.16 equivalents of alkali per mol of Hb present. Table VII of Adair (13) indicates 1.23 as the corresponding value for human cell contents. If the hemoglobin content of the cells in each case is 30 millimols per kilo of water, the concentrations of $[\text{BP}]_c$ will be 64.8 and 36.9 milli-equivalents per kilo of water, respectively. From differentiation of Equation 10 of Van Slyke, Wu, and McLean (given above) we calculate that the change, Δr , in the distribution ratio, caused by a given change, $\Delta [\text{BP}]_c$, in $[\text{BP}]_c$, other values in the equation being constant, will be:

$$\Delta r = \frac{-\Delta [\text{BP}]_c}{2 ([\text{B}]_c - [\text{BP}]_c)}$$

In both human and horse blood $[\text{B}]_c$ may be taken as 175 milli-equivalents, and $[\text{BP}]_c$ as 15, without significant error for our present calculation. The $\Delta [\text{BP}]_c$ caused by the change from horse to human blood is $36.9 - 64.8 = -27.9$ milli-equivalents. Hence

$$\Delta r = \frac{+27.9}{320} = +0.087$$

From the difference in base-binding power of the cell colloids we thus estimate that the r_{Cl} and r_{HCO_3} should be about 0.09 higher in human than in horse blood at the same pH.

Inspection of Fig. 1 shows that in fact the Cl and HCO_3 distribution ratios for both normal and pathological human blood lie above the curves found by Van Slyke, Hastings, Murray, and Sendroy (2) for horse blood, and by margins averaging not far from the above estimated 0.09. In the bloods from normal subjects

and patients with arthritis and cardiac disease there is an extreme variation of about ± 0.05 in either r_{Cl} or r_{HCO_3} at any given pH_s : such variation is to be expected from the possible variations in all

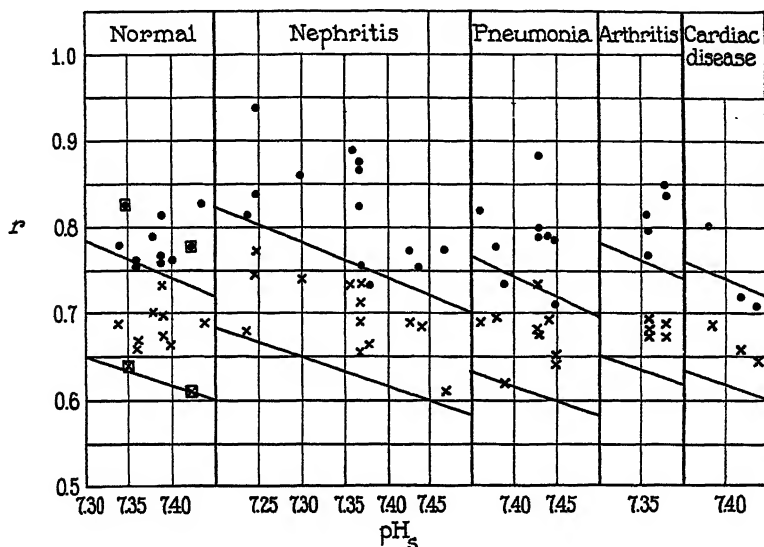


FIG. 1. Value of the chloride and bicarbonate distribution ratios in the oxygenated blood of normal and diseased human subjects.

- indicates $r_{\text{HCO}_3} = \frac{\text{BHCO}_3 \text{ per kilo H}_2\text{O in cells}}{\text{BHCO}_3 \text{ per kilo H}_2\text{O in serum}}$.
 x indicates $r_{\text{Cl}} = \frac{\text{Cl per kilo H}_2\text{O in cells}}{\text{Cl per kilo H}_2\text{O in serum}}$.

□ and ⊠ are data of Dill, van Caulaert, Hurxthal, Stoddard, Bock, and Henderson (14) on normal men.

The solid curves represent the mean values for the two ratios obtained in oxygenated horse blood by Van Slyke, Hastings, Murray, and Sendroy (2). The upper curve in the diagram for each group represents r_{Cl} , the lower r_{HCO_3} .

the factors that influence r . At any given pH_s , there is no marked difference between normal and pathological bloods in the r_{Cl} and r_{HCO_3} values; in the nephritis and pneumonia blood the variation at given pH_s is somewhat greater, about ± 0.07 , and in addition the total range of r values is further extended by the greater

range in pH_s .¹ That the r variation at given pH_s is not more markedly greater than in normal men may be attributed to a fair degree of constancy in the two single factors, *the hemoglobin concentration in the cells* and *the total base of the serum*, which are most important in fixing the value of r .²

It is of interest that the relation of r_{Cl} to r_{HCO_3} is the same in the blood of normal and pathological individuals. As the average of our determinations on human blood, we find

$$\frac{[\text{Cl}]_c}{[\text{Cl}]_s} : \frac{[\text{BHCO}_3]_c}{[\text{BHCO}_3]_s} = 0.87$$

In horse blood Van Slyke, Hastings, Murray, and Sendroy (2) found the ratio of r_{Cl} to r_{HCO_3} to be 0.81. The difference between chloride and bicarbonate distributions is presumably attributable to factors which affect the activity coefficients of Cl and HCO_3 anions in the cells, and which we have thus far been unable to identify.

CONCLUSIONS.

The distribution of chloride and bicarbonate between cells and serum has been studied in human venous blood. The distribution ratios $[\text{cell Cl}] : [\text{serum Cl}]$ and $[\text{cell BHCO}_3] : [\text{serum BHCO}_3]$ have been found to average several per cent higher than in horse blood at the same pH_s . The difference approximates that calculated by Equation 10 of Van Slyke, Wu, and McLean (1) from the lesser base-binding power of the colloid constituents of human cells, indicated by the analyses of Adair (13).

Studies of blood from patients with nephritis (severe but not uremic), cardiac disease, pneumonia, and acute arthritis, representing a fairly wide range of pathological conditions, failed to reveal evidence that in any of these conditions the mechanism

¹ Henderson, Bock, Dill, Hurxthal, and van Caulaert (15) have recently published analyses of blood from two nephritic patients in extremis. The pH_s values were much more depressed, to the neighborhood of 7.00, than in any of our cases, and the r values correspondingly higher, r_{Cl} being 0.92 in venous blood, 0.90 in arterial, while r_{HCO_3} exceeded unity.

² The much more numerous analyses of blood in various pathological conditions by Peters and his collaborators (16) indicate that even in disease it is rather rare for the serum total base or the ratio (O_2 capacity): (cell volume) to deviate by more than 10 per cent from the normal range.

controlling the electrolyte distribution becomes qualitatively altered, or that very marked quantitative deviations from the normal distribution ratios occur, other than the predicted r changes due to blood pH variation. The only definite quantitative deviation from the normal was a tendency for r_{HCO_2} values to be somewhat higher in part of the nephritic bloods.

The failure of the r_{Cl} and r_{HCO_2} ratios to deviate in the pathological bloods more markedly from the ratios observed at the same pH, in normal blood appears attributable to the facts that the two factors which are theoretically most important in determining the values of the ratios at any given pH are the total base concentration in the serum and the hemoglobin concentration in the cells, and that neither of these is liable to great fluctuations in the conditions studied.

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TABLE I.
Normal Subjects.

Subject.	Material.	H ₂ O content.		pH _s		[CO ₂] mM per kg. H ₂ O	CO ₂ tension. mm.	[H ₂ CO ₃] mM per kg. H ₂ O	[BHC0 ₃] mM per kg. H ₂ O	[Cl] m.-eq. per kg. H ₂ O	HbO ₂ content. mM per l. blood	HbO ₂ capacity. mM per l. blood	Oxygenation of Hb. per cent	Total base. m.-eq. per kg. H ₂ O	[BHC0 ₃] [HCO ₃] ⁻		[Cl] _s [Cl] _s ⁻	
		gm. per cc.	gm. gm.	Electrometric.	Colorimetric.										Found.	Calculated to 100 per cent oxygenation.	Found.	Calculated to 100 per cent oxygenation.
A. B. H.	Serum.	0.938	0.912	7.38	7.41	32.95	50.2	1.64	31.31	108.4	4.66	9.22	50.6		0.829	0.790	0.738	0.699
			0.691			27.75	50.2	1.78	25.97	80.1								
J. M.	Serum.	0.934	0.909	7.36	7.38	32.67	52.1	1.70	30.97	110.8					0.811	0.763	0.716	0.668
			0.670			26.94	52.1	1.84	25.10	79.3	3.63	9.88	36.8					
W. N.	Serum.	0.933	0.919	7.39	7.46	31.09	47.8	1.56	29.53	108.9					0.798	0.768	0.727	0.697
			0.678			25.26	47.8	1.69	23.57	79.2	5.66	8.95	63.2					
G.	Serum.	0.931	0.915	7.44		28.54	38.1	1.25	27.29	111.5				173.4	0.852	0.828	0.711	0.687
			0.685			24.60	38.1	1.35	23.25	79.3	6.41	8.92	71.8					
C. D.	Serum.	0.934		7.40		33.67	49.0	1.60	32.07	109.1				170.0	0.866	0.812	0.768	0.714
			0.690			29.52	49.0	1.73	27.79	83.8	3.27	9.99	32.8					
L. L.	Serum.	0.911	7.39			34.24	51.1	1.67	32.57	104.3				178.5	0.862	0.815	0.775	0.728
			0.668			29.84	51.1	1.81	28.03	80.8	3.93	9.25	42.5					

G. S.	Serum. Cells.	0.917 0.680	7.34		35.55 29.81	59.1 59.1	1.93 2.09	33.62 27.82	106.2 77.6	4.05	10.95	37.0		0.828	0.781	0.731	0.684
W. N.	Serum. Cells.	0.916 0.683	7.39		32.57 26.40	48.6 48.6	1.59 1.72	30.98 24.68	107.1 76.3	4.81	9.36	51.4	175.6	0.797	0.758	0.712	0.673
F.	Serum. Cells.	0.913 0.685	7.36		34.00 27.73	54.1 54.1	1.77 1.91	32.23 25.82	104.4 73.1	4.25	9.68	43.9		0.801	0.758	0.700	0.657
Maximum.....			7.44												0.828		0.728
Minimum.....			7.36												0.758		0.657
Mean.....			7.38												0.786		0.689

TABLE II.
Nephritic Subjects.

Subject.	Material.	H ₂ O content.		pH _s		[CO ₂] mM per kg. H ₂ O	CO ₂ tension. mm.	[H ₂ CO ₃] mM per kg. H ₂ O	[BHC0 ₃] mM per kg. H ₂ O	[Cl] m-equiv. per kg. H ₂ O	HbO ₂ content. mM per L. blood	HbO ₂ capacity. mM per L. blood	Oxygenation of Hb. per cent	Total base. m-equiv. per kg. H ₂ O	Serum proteins. gm. per kg. H ₂ O	[BHC0 ₃] [BHC0 ₃] [Cl] [Cl] _s		Calculated to 100 per cent oxygenation.	Found.	Calculated to 100 per cent oxygenation.
		gm. per cc.	gm. per gm.	Electrometric.	Colorimetric.											Found.	Found.			
Sime.	Serum. Cells.	0.923	0.688	7.25		15.55 14.94	31.5	1.03 1.12	14.52 13.82	110.9 86.9		3.18	4.08	78.0	171.7	63.7	0.953	0.939	0.784	0.770
		0.922	0.683	7.24		18.41 15.56	38.0	1.24 1.35	17.17 14.21	101.4 70.4		3.38	4.29	78.8	163.6	66.7	0.829	0.815	0.694	0.680
Giaq.	Serum. Cells.	0.943	0.672	7.43		29.27 24.55	39.9	1.30 1.41	27.97 23.14	107.1 79.8		3.34	6.11	54.7	166.5		0.828	0.773	0.745	0.690
		0.928	0.672	7.37		29.49 23.25	46.0	1.50 1.63	27.99 21.62	110.7 74.3		6.59	8.37	78.8	166.8	80.3	0.772	0.756	0.671	0.655
Carr.	Serum. Cells.	0.935	0.682	7.47		28.44 22.40	35.3	1.15 1.25	27.29 21.15	106.8 65.5		5.46	5.58	97.8	162.6	43.6	0.776	0.774	0.612	0.610
		0.947	0.671	7.38		27.46 21.36	41.8	1.37 1.48	26.09 19.88	114.3 78.8		3.18	5.22	61.0	165.5		0.762	0.732	0.690	0.660

Ols.	Serum. Cells.	0.941 0.685	7.36	23.65 20.26	37.7 1.2322.42 1.3418.92	109.5 77.4	4.44 5.80	76.5	161.7 58.60.843	0.8250.707	0.689
Cor.	Serum. Cells.	0.946 0.713	7.44	34.62 28.33	46.2 1.5133.11 1.6426.69	108.0 79.5	3.13 7.64	41.0	159.3 55.90.806	0.7560.736	0.686
Cunn.	Serum. Cells.	0.937 0.641	7.36	29.55 27.46	47.0 1.5423.01 1.6625.80	109.8 84.0	4.46 7.62	58.5	0.920 0.8880.765	0.733	0.733
Val.	Serum. Cells.	0.948 0.689	7.37	29.83 27.97	46.6 1.5228.31 1.6526.32	112.1 86.1	2.39 8.34	28.7	0.930 0.8750.768	0.713	0.713
Clar.	Serum. Cells.	0.933 0.703	7.37 7.30	29.20 23.18 21.39	49.8 1.6330.27 1.7627.44	101.4 77.8	4.88 8.92	54.7	0.906 0.8710.768	0.733	0.733
Wohl.	Serum. Cells.	0.932 0.696	7.30	23.18 21.39	41.9 1.3721.81 1.5219.87	89.4 70.8	1.60 5.60	28.6	145.0 58.10.911	0.8610.791	0.741
McK.	Serum. Cells.	0.935 0.699	7.25	15.86 13.70	32.1 1.0514.81 1.1412.56	97.8 73.7	4.05 4.63	87.4	144.5 57.10.847	0.8390.754	0.746
Maximum.....			7.44							0.939	0.770
Minimum.....			7.25							0.732	0.610
Mean.....			7.35							0.824	0.700

Case Cunn. was the arteriosclerotic type of Bright's disease; Cor. was an acute hemorrhagic case which subsequently recovered. The other cases were of chronic hemorrhagic Bright's disease (Addis' classification) in advanced stages with marked functional impairment, but none was in actual uremia.

Ma.	Serum. Cells.	0.923	0.986	7.36	7.36	33.59	52.9	1.73	31.86	102.2	4.34	10.33	42.0	0.862	0.820	0.734	0.690
						29.21	52.9	1.87	27.34	75.0							
Cot.	Serum. Cells.	0.942	0.983	7.45	7.44	34.71	45.4	1.48	33.23	91.7	4.33	8.16	53.0	0.750	0.710	0.688	0.648
						26.53	45.4	1.61	24.92	63.1							
Hir.	Serum. Cells.	0.934	0.983	7.38	7.38	31.62	48.1	1.57	30.05	111.2	4.40	8.47	52.0	0.771	0.734	0.657	0.620
						24.86	48.1	1.70	23.16	73.2							
	Maximum.....														0.887		0.732
	Minimum.....														0.710		0.620
	Mean.....														0.788		0.674

TABLE IV.
Acute Arthritis and Cardiac Disease.

Subject.	Material.	H ₂ O content.	pH _s electrometric.	[CO ₂]	CO ₂ tension.	[B _s CO ₂]	[BHC0 ₃]	[Cl]	HbO ₂ content.	HbO ₂ capacity.	Oxygenation of Hb.	[BHC0 ₃] _s [BHC0 ₃] _s		[Cl] _c [Cl] _s		
												Found.	Calculated to 100 per cent oxygenation.	Found.	Calculated to 100 per cent oxygenation.	
Arthritis.																
Dob.	Serum. Cells.	gm. per cc.	gm. per gm.	mm. per 100. H ₂ O	mm.	mm. per 100. H ₂ O	mm. per 100. H ₂ O	mm.-eq. per 1. H ₂ O	mm. per 1. blood	mm. per 1. blood	per cent	0.810	0.764	0.734	0.680	
		0.928	0.664	34.46 28.40	54.9 54.9	1.79 1.94	32.67 26.46	105.2 77.2	3.05	7.76	39.3					
Stil.	Serum. Cells.	0.932	0.704	33.25 28.27	52.9 52.9	1.73 1.87	31.52 26.40	108.4 79.3	3.52	8.10	43.5	0.838	0.795	0.732	0.689	
		0.921	0.702	29.20 26.18	44.4 44.4	1.45 1.57	27.75 24.61	109.7 80.6	2.88	8.04	35.9	0.887	0.836	0.734	0.683	
Lin.	Serum. Cells.	0.931	0.712	30.78 27.10	49.1 49.1	1.61 1.74	29.17 25.36	109.2 79.6	2.54	8.80	28.9	0.870	0.816	0.729	0.675	
		0.932	0.681	35.53 32.17	54.0 54.0	1.76 1.91	38.77 30.26	106.2 76.1	3.59	9.13	39.4	0.896	0.849	0.717	0.670	
Coh.	Serum. Cells.													0.849		0.689
														0.769		0.670
														0.812		0.640

Cardiac disease.

Gebr.	Serum. Cells.	0.937		7.44	34.00	45.4	1.48	32.52	106.9				0.735	(0.707)	0.673	(0.644)
			0.714		25.53	45.4	1.61	23.92	71.9							
Caff.	Serum. Cells.	0.925		7.42	33.22	46.4	1.52	31.70	107.8				0.762	0.738	0.681	0.657
			0.688		25.78	46.4	1.64	24.14	73.4	6.42	9.02	71.2				
Schw.	Serum. Cells.	0.927		7.38	33.65	51.2	1.67	31.98	108.0				0.835	0.800	0.710	0.685
			0.700		28.52	51.2	1.81	26.71	76.7	4.61	10.00	46.1				

THE OXIDATION OF DIXANTHYDRYL UREA, A MICRO METHOD FOR DETERMINING UREA.

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The method described by Kiech and Luck (1) for the estimation of urea suffers from three undesirable features. Like many gravimetric methods it requires special apparatus and the expenditure of much time. A fairly large amount of urea, somewhat more than 0.5 mg., is also needed. This quantity is contained in about 20 cc. of the tungstic acid filtrate from normal blood and tissues and only too often it was found inconvenient to spare so large a quantity. We were therefore driven to exercise further economies in time and material.

In the course of his extensive description of xanthidrol, Fosse (2) pointed out that this substance is readily oxidized to xanthone. From this it occurred to us to investigate the action of oxidizing agents upon dixanthidryl urea in the hope of developing a quantitative volumetric method for the estimation of urea. A few preliminary experiments showed that dixanthidryl urea dissolved in strong acids to give fluorescent solutions of a brilliant canary-yellow color, which on oxidation with potassium permanganate, became colorless. As the end-point could be determined quite readily under the optimum conditions, we were enabled to utilize these facts as the basis of a quantitative method. Before describing the procedure, it would be well to point out that oxidation appears to proceed far beyond the formation of xanthone.

Method.

2 to 5 cc. of the tungstic acid filtrate (1, 3) contained in a 15 cc. centrifuge tube are diluted to 5 cc. with water.¹ (For normal

¹We have been employing Eastman's preparation of xanthidrol. We have not succeeded in obtaining complete solution of the substance in methyl alcohol, although Fosse appears to have done so. The solution

blood use 5 cc. of filtrate.) 5 cc. of glacial acetic acid and 0.5 cc. of 10 per cent xanthidryl in methyl alcohol² are added. The contents must be intimately mixed. After 1 hour the tube is centrifuged, the supernatant fluid is decanted off, and the precipitate of dixanthidryl urea washed once with 5 cc. of a saturated solution of dixanthidryl urea in methyl alcohol. This serves to remove the excess xanthidryl. The tube is again centrifuged and the fluid removed by decantation. The excess of alcohol which clings to the precipitate and the wall of the tube is removed by drying. A few minutes at 100° is sufficient for this purpose. 8 cc. of cold 1:1 sulfuric acid (equal volumes of water and concentrated acid) are now added.³ The residue dissolves slowly to form a yellow solution which is washed with 32 cc. of water into an evaporating basin of white porcelain, 10 cm. in diameter. The contents are heated to 70–75° and titrated at this temperature with 0.05 N potassium permanganate contained in a 5 cc. burette. It is advisable to carry out the titration in a bright, white light having at hand a second porcelain basin containing 40 cc. of water. The latter is of great assistance as a standard and is used for purposes of comparison as the end-point is neared. It should not be artificially illuminated. If the basin containing the solution of dixanthidryl urea be removed from beneath the burette and placed beside the standard, or between two such standards after each addition of permanganate the end-point may readily be determined. The fluid must be titrated until the last tint of yellow has disappeared.

It is necessary to maintain an acid concentration of 10 per cent (by volume). Since the latter changes by dilution during the titration, it is advisable to add, when near the end-point, an addi-

used in this work was prepared by suspending 10 gm. of xanthidryl in 90 gm. of pure methyl alcohol, shaking at intervals during 3 days, and filtering.

² Since this method is applicable to the estimation of dixanthidryl urea however prepared, it is apparent that the use of other protein precipitants or conditions of precipitations are permissible,—provided only that the subsequent formation of dixanthidryl urea be quantitative and productive of a pure, crystalline ureide.

³ Complete solution of the dixanthidryl urea is essential. It is advisable to pulverize the precipitate by grinding it against the bottom of the centrifuge tube with a stirring rod before adding the sulfuric acid.

tional amount of the 1:1 sulfuric acid equal to one-fourth of the volume of permanganate employed.

Not more than 3 cc. of permanganate should be employed in the titration. Somewhat beyond this point the relationship between the amounts of permanganate and urea ceases to be linear. Below this upper limit 1 cc. of 0.05 N KMnO_4 = 0.093 mg. of urea.

For the estimation of urea in urine, dilute 1 cc. of urine in a volumetric flask to 100 cc. Transfer 1 cc. of the diluted urine to a 15 cc. centrifuge tube, add 4 cc. of water, 5 cc. of glacial acetic acid, and 0.5 cc. of the methyl alcohol solution of xanthydril. Continue with the procedure given above.

EXPERIMENTAL.

Preparation of Dixanthydril Urea.

To 40 cc. of 0.20 per cent urea were added 140 cc. of glacial acetic acid and 20 cc. of 10 per cent xanthydril in methyl alcohol. The latter was added in 5 cc. portions at 5 minute intervals. 1 hour and 25 minutes after the last addition, the mixture was centrifuged and the crystalline precipitate washed with 100 cc. of ethyl alcohol and again centrifuged. The residue was washed onto a suction filter with 50 cc. of ethyl alcohol and dried at 60°. The product was employed in the first few experiments and for preparation of the saturated solution of dixanthydril urea in methyl alcohol.

Optimum Concentration of Sulfuric Acid.

33.3 mg. of dixanthydril urea were dissolved in 100 cc. of 1:1 sulfuric acid. 3 cc. portions were diluted with water and sulfuric acid to volumes of 40 cc. and sulfuric acid concentrations of 5 to 50 per cent. They were titrated at 60–70° with 0.059 N potassium permanganate. It was found that the titration value is dependent upon the concentration of sulfuric acid and is indeed a linear function of the concentration between 5 and 30 per cent. For future work it was decided to employ an acid concentration of 10 per cent. The color given by the dixanthydril urea in 5 per cent sulfuric acid was relatively faint. It is also obvious that a

maximum depth of color with a maximum titration value is desirable. Nevertheless it was not permissible to use very high concentrations of sulfuric acid. In an acid concentration of 50 per cent the fluid became brownish during titration, the end-point was very obscure and, indeed, charring appeared to have proceeded. In order to minimize the possibility of such a happening, the relatively low acid concentration of 10 per cent was selected. This was used in all later work. Only on completion of the experiments described in this paper was it discovered that in view of the approximately linear relationship between acid concentration and cc. of permanganate the use of at least 20 per cent sulfuric acid would probably have been permissible. As the titrations have proceeded satisfactorily under the conditions described we have not seen fit to attempt this change, although the delicacy of the method might be thereby enhanced.

Volume of Sulfuric Acid.

Although the quantity of permanganate required for the oxidation of a given amount of dioxanthryl urea is a function of the concentration of sulfuric acid it is independent of the volume.

Five portions of dioxanthryl urea solution, each of 3 cc., were each diluted with 12 cc. of water. Increasing amounts of 10 per cent sulfuric acid (15 cc., 25 cc., 25 cc., 40 cc., 40 cc.) were added. The solutions were then titrated at 70–75° with 0.059 N KMnO_4 . The following titration values were obtained: 1.34 cc., 1.29 cc., 1.32 cc., 1.28 cc., and 1.33 cc. respectively.

The experiment was repeated with 4 cc. portions of dioxanthryl urea solution to which were added 16 cc. of water and 5 to 40 cc. of 10 per cent sulfuric acid. Titrations were made with 0.0501 N KMnO_4 at 70–75°. Fourteen samples were run. All values fall between 2.05 and 2.19 cc. of potassium permanganate.

Oxidation of Varying Quantities of Dioxanthryl Urea.

66.6 mg. of dioxanthryl urea were dissolved in 1:1 sulfuric acid and made up to a volume of 200 cc. Ten portions of increasing size (2 cc. to 8 cc.) were diluted with water and sulfuric acid to final volumes of 40 cc. and acid concentration of 10 per cent. Titrations were made at 70–75°.

It was found that up to 2 mg. of dioxanthryl urea the quantity

of potassium permanganate required for the oxidation is a linear function of the amount of ureide present. All values for the urea equivalent (in mg.) of 1.0 cc. of 0.05 N potassium permanganate fell between 0.091 and 0.096. The average was 0.093.

Application of Method to Solutions of Urea.

An aqueous solution of urea containing 100 mg. of urea per liter was prepared. Portions of the solutions contained in small

TABLE I.
Estimation of Urea.

Urea solution.	0.0501 N KMnO ₄ .	Urea found.	Urea calculated.
cc.	cc.	mg.	mg.
1.0	1.04	0.099	0.100
1.0	1.10		
1.5	1.66	0.150	0.150
1.5	1.57		
2.0	2.06	0.198	0.200
2.0	2.19		
2.5	2.70	0.247	0.250
2.5	2.62		
3.0	3.30	0.305	0.300
3.0	3.26		
3.5	3.32	0.313	0.350
3.5	3.39		
4.0	3.96	0.351	0.400
4.0	3.58		

centrifuge tubes were made up to 5 cc. with water. The urea was precipitated as dixanthhydryl urea by the addition of 5 cc. of glacial acetic acid and 0.5 cc. of xanthhydryl solution. The washed precipitates were estimated by oxidation with potassium permanganate, according to the procedure already described. The results are presented in Table I.

Estimation of Urea in Blood.

Six portions of rabbit blood, each of 2 cc., were treated as follows: To the first was added 14 cc. of water, to the second 13 cc. of water and 1 cc. of urea solution (0.25 mg. per cc.), to the third 12 cc. of water and 2 cc. of urea, to the fourth 11 cc. of water and 3 cc. of urea, to the fifth 10 cc. of water and 4 cc. of urea, and to the sixth 8 cc. of water and 6 cc. of urea. Each received 2 cc. of sodium tungstate (10 per cent) and 2 cc. of sulfuric acid (0.66 N). Of the six filtrates which were then obtained, 2 and 3 cc. portions of each were treated with water, glacial acetic acid, and xanthidrol according to the routine procedure.

The experiment was repeated upon the blood of another rabbit.

The results of both experiments are summarized in Table II.

TABLE II.
Recovery of Urea from Blood.
Urea in 2 cc. of blood.

Experiment 1.		Experiment 2.	
Found.	Calculated.	Found.	Calculated.
mg.	mg.	mg.	mg.
1.00		0.50	
1.29	1.25	0.74	0.75
1.55	1.50	1.06	1.00
1.82	1.75	1.28	1.25
2.06	2.00	1.56	1.50
2.47	2.50	2.03	2.00

Each value represents the average of two determinations, one of which was obtained from 2 cc. of filtrate, and the other from 3 cc.

Estimation of Urea in Animal Tissue.

An adult female rat was killed by stunning and deeply incising the thorax. The blood was collected over potassium oxalate and the liver and muscles of one hind limb excised and frozen with liquid air. The powdered tissues, 4 gm. samples, were treated with tungstic acid as described in a preceding paper (1). Urea estimations were made upon portions of each filtrate by the volumetric method described in this paper. These results, together with those from a second rat, are presented in Table III.

Use of Tanret's Reagent as a Protein Precipitant.

Kiech and Luck (1) reported that the application of their method to muscle gave impure, yellowish precipitates of the ureide. In the course of the experiments which have been summarized in Table III, trouble was again experienced with the muscle preparations. The unwashed dioxanthidryl urea obtained after the first centrifugation was somewhat discolored. Practically all of the colored impurity appeared to dissolve in the methyl alcohol for the washed product was quite white. Nevertheless when the attempt to dissolve the dried precipitates in 1:1 sulfuric acid was made, there appeared to be a small portion of the muscle samples which resisted solution.

TABLE III.
Urea Content of Rat Blood, Liver, and Muscle.

Tissue.	Volume of filtrate used.		0.05 N KMnO ₄ .		Urea in 100 gm. of tissue.	
	Rat 1.	Rat 2.	Rat 1.	Rat 2.	Rat 1.	Rat 2.
	cc.	cc.	cc.	cc.	mg.	mg.
Blood.....	2	3	0.41	0.64	19.1	19.8
"	3	5	0.64	0.99	19.8	18.4
Liver.....	2	5	0.38	1.00	17.7	18.6
"	3	5	0.60	0.99	18.6	18.4
Muscle.....	2	5	0.28	0.94	13.0	17.5
"	3	5	0.49	0.94	15.2	17.5

It seemed advisable therefore to check the tungstate procedure by the use of another protein precipitant. For this purpose we employed Tanret's reagent⁴ as modified by Fosse, Robyn, and François (4).

Duplicate 4 gm. samples of frozen, powdered liver and muscle were weighed out. Two rats had to be employed to supply enough liver tissue, the latter being intimately mixed before weighing. The first of each pair of tissue samples was treated with 28 cc. of ice-cold water, 4 cc. of ice-cold sodium tungstate (10 per cent), and 4 cc. of ice-cold sulfuric acid (0.66 N). The second sample of each pair received 32 cc. of water, and 4 cc. of Tanret's

⁴ Tanret's reagent consists of: mercuric chloride 2.71 gm., potassium iodide 7.20 gm., and glacial acetic acid 66.6 cc.; diluted with water to 100 cc.

reagent. Of the four filtrates which were then obtained, 3 cc. and 5 cc. portions of each were treated with glacial acetic acid and xanthydrol. The rest of the procedure was that described in the first portion of this paper.

This experiment was performed a second and third time. In a fourth experiment a pregnant female rat (18th day of gestation) was employed and analyses were made upon the fetus as well. This animal had received urea by subcutaneous injection 25 minutes before. The results are summarized in Table IV.

TABLE IV.
Tanret's Reagent as Protein Precipitant.

Tissue.	Protein precipitant.	Volume of filtrate used.	Urea content per 100 gm. of tissue.			
			Experiment 1.	Experiment 2.	Experiment 3.	Experiment 4.
		cc.	mg.	mg.	mg.	mg.
Liver.....	Tungstic acid.	3	17.5	20.6	27.6	83.7
"	" "	5	16.5	19.2	25.6	
"	Tanret's reagent.	3	16.9	21.5	27.0	80.5
"	" "	5	17.5	21.2	25.9	
Muscle.....	Tungstic acid.	3	17.6	18.8		54.5
"	" "	5	16.6	17.3	24.8	
"	Tanret's reagent.	3	22.2	21.2	26.3	88.3
"	" "	5	23.3	21.0	26.3	
Whole fetus.....	" "	5				54.3
" "	" "	5				58.7

In Experiment 4, the animal received urea by subcutaneous injection 25 minutes before being killed.

From these it may be concluded that tungstic acid and Tanret's reagent are equally satisfactory for the precipitation of the proteins of the liver and fetus. Identical urea values are obtained.

When applied to muscle, however, tungstic acid permits the passage into the filtrate of some product which contaminates the dioxanthidryl urea ultimately obtained, interferes with its solution in 1:1 sulfuric acid, and leads to low titration values.

Believing that it might be possible to precipitate the interfering substance by adding Tanret's reagent to tungstic acid filtrates of muscle, we made several experiments with that end in view. The addition of 0.1 to 0.3 cc. of Tanret's reagent gave no precipi-

tate. It was found that the subsequent addition of glacial acetic acid and xanthidrol gave precipitates of dixanthidryl urea which were less contaminated, and which dissolved more readily in 1:1 sulfuric acid, but complete solution of the precipitates was not obtained. The permanganate titration values were still inclined to be lower than those which followed the use of Tanret's reagent only.

Were it not for the need of estimating substances other than urea in the protein-free tissue extracts we would be inclined to use Tanret's reagent as the protein precipitant. We have however been mindful of the value of using a reagent which would be of general utility in tissue analysis. Thus we have found Tanret's reagent quite unsuitable if the estimation of amino acids be desired. It is nevertheless necessary to use this reagent as the protein precipitant in estimating the urea content of muscle.

The presence of glycogen in the tissue extracts does not interfere in the determination of urea by this method.

SUMMARY.

Dixanthidryl urea dissolves in sulfuric acid to give fluorescent yellow solutions which are rendered colorless by oxidation with potassium permanganate. These facts have been employed in the development of a volumetric method for the estimation of urea. 0.1 mg. may be estimated with an experimental error of about 5 per cent.

Alan Archer McCray and Frank Hilton Smith assisted in part of this work.

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Addendum.—Since this work was submitted for publication, a paper by F. Beattie appeared (*Biochem. J.*, 1928, xxii, 711) in which dixanthidryl urea is estimated colorimetrically by comparison of the yellow solution, here described, against a standard.

A STUDY OF THE ESTIMATION OF CHLORIDE IN BLOOD AND SERUM.

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Many modifications have been suggested for the determination of chloride in blood and other body fluids. The simplest and most thoroughly studied is a method proposed by Van Slyke (1) involving ashing of serum with concentrated nitric acid in the presence of silver nitrate to prevent volatilization of the chloride.

In making a preliminary study of Van Slyke's method before using it for a series of analyses, we attempted to cut down the period of digestion (2 hours on a water bath) by boiling the mixture gently over a micro burner until the solution was clear. When this was done it was noted that the results tended to be higher than those obtained by the regular Van Slyke procedure. The silver nitrate in nitric acid used by Van Slyke forms an immediate voluminous precipitate, protein and silver chloride, when added to serum. In order to diminish the possibility of occlusion of chloride or silver, it was thought that the reagents might better be added separately. Aqueous silver nitrate was therefore prepared. It was made 3 times as strong as Van Slyke's standard so that the total volume of the solution could be kept small. The procedure used was: 1 cc. of serum was measured into a large test-tube (approximately 25×200 mm.), 1 cc. of the standard aqueous silver nitrate solution added, and the tube shaken; then 3 cc. of concentrated nitric acid were introduced, and, after being mixed, the contents were digested. Some digestions were carried out by heating the tubes in a boiling water bath for 2 hours and others by boiling the solutions gently over a micro burner for 15 to 20 minutes. After a few determinations were carried out and good results obtained, a series of comparisons was made with (1)

the regular Van Slyke procedure with digestion for 2 hours and 16 hours, (2) aqueous silver nitrate and nitric acid with digestion in a water bath or over a free flame, (3) the Whitehorn method¹ (2).

Besides these micro methods, determinations with two macro methods were run in the hope that these might yield more accurate results and thereby constitute standard determinations with which to compare the results of the micro procedures. The methods were: (1) the Parr bomb method, which consists of burning dried organic material mixed with sodium peroxide in a Parr bomb and then determining the chloride gravimetrically. 10 cc. of serum were evaporated to dryness in a Parr bomb, the residue mixed with 10 gm. of sodium peroxide, and the determination carried out as described by Parr (3). (2) Alkaline ashing: 10 cc. of serum were evaporated to dryness in a platinum dish after the addition of from 0.5 to 1 gm. of sodium carbonate. The dry residue was carefully ashed by the Stolte (4) procedure. The white ash was dissolved in about 100 cc. of water, filtered, and the chloride precipitated by the regular procedure with nitric acid and silver nitrate. The precipitated silver chloride was filtered in a Gooch crucible, dried, and weighed. Table I gives the results of these series of analyses.² It may be seen that good agreement was obtained with all of the procedures except the Van Slyke which yielded results lower than the others.

When these results were called to Dr. Van Slyke's attention he carried out a series of analyses on human sera and failed to find such differences. Through his cooperation, Dr. Alma Hiller, his assistant, came to our laboratory and carried through a series of analyses with us. We then learned for the first time that Van Slyke's laboratory had used 50 cc. flasks instead of tubes on account of the convenience in titrating. A comparison was, therefore, made with both flasks and tubes with the Van Slyke and the aqueous silver nitrate procedures. The results are shown in Table II. It will be observed that the use of tubes with the Van Slyke

¹ 1 cc. of our standard aqueous silver nitrate solution was used instead of the silver standard recommended. No correction was subtracted from the thiocyanate used to titrate the excess of silver.

² The macro determinations reported in this paper were made in duplicate, the micro determinations in triplicate, except Experiments 4 and 5 on whole blood which were made in duplicate.

method yielded lower values than those obtained with flasks. However, the aqueous silver nitrate yielded still higher results. With the latter procedure no difference was noted between the results obtained with tubes or flasks. Thus it would appear that the silver nitrate and nitric acid should be added separately.

TABLE I.
Chlorides in Serum (Mg. of NaCl per 100 Cc.).

Sample No.....	Dog serum.				Human serum.		Horse serum.	Beef serum.
	6	7	8	9	1	2	2	1
Parr bomb.....	692	669	678	753	632	624		
Alkaline ashing.....		669	683	751				
Van Slyke, 2 hrs.....	660	632	660	672	598	572	590	591
“ “ overnight.....	670	660	669	715	607	597	618	
Aq. Ag, boiled 20 min.....	691	661	680	750	624	622	619	595
“ “ water bath 2 hrs..	688	661	675	753	624	621	618	
Whitehorn.....	688	667	690	756	639	624		

TABLE II.
Comparison of Tubes and Flasks (Mg. of NaCl per 100 Cc.).

	Human Serum 3.		Dog Serum 10.		Dog Serum 11.		Horse Serum 2.		Dog Whole Blood 3.	
	Flasks.	Tubes.	Flasks.	Tubes.	Flasks.	Tubes.	Flasks.	Tubes.	Flasks.	Tubes.
Van Slyke, 2 hrs.	610 H	603 B	626 H	617 B	740 B	676 B	611 H	611 B		471 * B
					738 H	699 H				
Aq. Ag, water bath 2 hrs....	621 “	623 “	623 “	623 “	754 B	751 B	615 “	613 “	486 B	484 “
					753 H	748 H				

B, run by Mr. Ball. H, run by Dr. Hiller.

* 16 hours.

A few experiments were carried out to study the effect of varying the time of digestion. The method of Osterberg and Schmidt¹ (5) was also used for comparison. Osterberg and Schmidt add dilute nitric acid and then silver nitrate and titrate in the presence of the precipitated protein and silver chloride. The results are given in Table III.

We obtained good agreement with all of the procedures except

the regular Van Slyke method. The end-point of the titration when the protein precipitate is present, as in the Osterberg and Schmidt method and in our undigested aqueous silver nitrate procedure, is quite poor. The turbid solution takes on a muddy appearance before the end-point is reached. Osterberg and Schmidt recommend a very fleeting end-point. A permanent end-point may be obtained by adding 1 or 2 more drops of thiocyanate solution. After digestion for 15 minutes with aqueous silver

TABLE III.
Chlorides in Serum (Mg. of NaCl per 100 Cc.).
Dog Serum.

Sample No.....	8		10		11		12		13	
	Flasks.	Tubes.	Flasks.	Tubes.	Flasks.	Tubes.	Flasks.	Tubes.	Flasks.	Tubes.
Van Slyke 2 hrs.....	660	626	617	738	676		666	638	634	627
Aq. Ag, water bath 2 hrs.	675		622	754	750			661		633
" " " " 30 min.	679		622		752					
" " " " 15 "	680		621				662	661	635	633
" " direct.....							668	668	645 p	642 p
								663 p		
Osterberg and Schmidt, direct.....	690		631		744		663	650	638	641
							656 p	638 p	629 p	629 p
Osterberg and Schmidt, digested.....							665		629	
							655 p			
Whitehorn.....	690		632		759		658		635	

p = permanent color at end-point.

nitrate and nitric acid, the protein is sufficiently digested so that the end-point of the titration is just as sharp as that obtained after a 2 hour digestion, and the duplicates agree well. After making these comparisons we feel that the sharper end-point obtained after a short digestion is much more preferable than the end-point obtained with the Osterberg and Schmidt procedure.

In Table IV will be found a series of analyses of whole blood. On account of the dark color produced by the precipitated hematin the titration without digestion is obviously impossible. Good

determinations were obtained when titrations were made on solutions digested long enough to remove the brown color. When tubes were used the brown color had disappeared after 40 minutes digestion in a boiling water bath but 1 hour's heating in flasks on top of a boiling water bath was insufficient. The Van Slyke

TABLE IV.
Chlorides in Whole Blood (Mg. of NaCl per 100 Cc.).
Dog Whole Blood.

Sample No.....	2	3	4		5	
			Flasks.	Tubes.	Flasks.	Tubes.
Alkaline ashing.....	532	489				
Van Slyke, overnight.....	515*	471*	501		482	
Aq. Ag, boiled 20 min.....	527*	482*				
“ “ overnight.....	526*	478*	509		484	
“ “ water bath 40 min.....			498 a 504 b	508		484
“ “ “ “ 1 hr.....					482	
“ “ hot plate 15 min.....			507			
“ “ water bath 20 min.....					474 a	475 a
Whitehorn.....	530	491	517		484	

a, solution brown. b, immersed in water bath.

* Determinations carried out in tubes only.

TABLE V.
Chlorides in Tissues (Mg. of NaCl per 100 Gm.).

Tissue.	Direct alkaline ashing.	Aq. Ag, boiled 30 min.	Van Slyke, 16 hrs.
Beef liver.....	134	130	125
“ muscle.....	125	118	108

procedure which involves digesting overnight yielded results slightly lower than the others.

Table V gives the results of two series of analyses on tissues. The Van Slyke procedure yielded results lower than those obtained by alkaline ashing. With aqueous silver nitrate it is doubtful if maximum results can be obtained. We hope to carry out further studies along this line.

DISCUSSION.

It appears that there are some dog sera and many human sera with which the Van Slyke method will yield maximum results, especially if flasks are used instead of test-tubes. On account of the poor results frequently encountered and the dependence of the method on the type of container used for the determination, the modification in which aqueous silver nitrate and nitric acid are used in separate solutions seems preferable. In our aqueous silver nitrate procedure the silver nitrate is added before the concentrated nitric acid because, if the nitric acid is added first, chloride may volatilize from the concentrated acid solution. However, Osterberg and Schmidt add diluted acid before adding the silver nitrate. As the Osterberg and Schmidt method yields good results even though the end-point is poor, it would seem that the element of greatest importance is that the protein should not be precipitated at the same time as the silver chloride.

The procedure which appears to be most convenient and satisfactory is the modification of Van Slyke's method as follows: To 1 cc. of serum or whole blood are added with mixing 1 cc. of standard silver nitrate (0.15 N) and then 3 cc. of concentrated nitric acid. The mixture is digested until the protein has been dissolved (15 minutes are required for serum and 40 minutes for whole blood when tubes are immersed in a boiling water bath. Flasks containing whole blood may be digested in about 15 minutes on a hot plate just below the boiling point). After digestion is sufficiently complete 6 cc. of 5 per cent ferric alum are added and the solution is cooled to room temperature or lower: the colder the solution the sharper the end-point.³ The solution is then titrated with 0.02 N ammonium thiocyanate with a micro burette and titration continued until 1 drop causes a color change which persists for about 1 minute at room temperature. The end-point is sharp. 0.02 to 0.04 cc. is subtracted from the titration to correct for the amount of thiocyanate required to give a suitable end-point in the presence of the silver chloride and thiocyanate, the nitric acid, and the water.

³ We have tried to sharpen the end-point still more by using acetone and alcoholic thiocyanate as recommended by Smirk (Smirk, F. H., *Biochem. J.*, 1927, xxi, 31) without observing any definite improvement.

SUMMARY.

Van Slyke's method for chloride in blood or serum is modified by use of aqueous silver nitrate and nitric acid as separate reagents instead of the silver nitrate dissolved in nitric acid.

A critical study of various procedures was made.

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Note by Donald D. Van Slyke and Alma Hiller.

By the courtesy of Professor Wilson we have had the opportunity to test his improvement on the open Carius method, for blood chlorides, and we are in entire agreement with him that the reliability of the method is increased by adding the AgNO_3 in water solution, before the nitric acid. With horse sera we have never found that this change caused any difference in results. But in two human sera out of fourteen the results were 1 and 3 milli-equivalents per liter respectively (6 and 18 mg. of NaCl per 100 cc.) lower when our original procedure was followed, of adding the AgNO_3 dissolved in concentrated HNO_3 to the serum. We were unable to identify the serum constituents responsible for the abnormal results in the two cases. One was a lipemic serum, but the other was not. The method as improved by Wilson and Ball appears to give entirely correct values for sera of all types.

The writers take advantage of this opportunity to call attention to an earlier paper on the subject that was overlooked when the former paper was published, in which the first successful application of the open Carius principle to blood was ascribed to Greenwald. Laudat (*J. pharm. et chim.*, 1917, xvi, 168) had several years before published a method in which the organic matter of blood was destroyed by boiling with KMnO_4 and HNO_3 in the presence of AgNO_3 , and the Cl was determined by titration of the excess Ag with NH_4CNS .

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THE HEMOLYTIC ACTION OF INORGANIC ACIDS.

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(Received for publication, June 8, 1928.)

In his monograph "The Erythrocyte and the Action of Simple Hemolysins," Ponder (1) states that very little satisfactory work has been done on the hemolytic action of acids and alkalies. Reference to Jacobs' review in Cowdry's "General Cytology" (2) and to Höber's and Bayliss' discussions of hemolysis and the permeability of the red blood cell (3, 4), as well as to the work on hemolysis by Stewart (5) and others, confirms the impression that the subject has received scant attention.

The present paper reports certain observations that have been obtained in a study of hemolysis by the following acids: hydrochloric, sulfuric, nitric, and phosphoric.

Two types of corpuscles have been employed in this study; namely, those of the dog and man. The procedure employed in determining the time required for complete hemolysis of a standard cell suspension has been based on the method of Ponder (6).

Preparation of Red Blood Cell Suspension.—The cell suspension is prepared as follows: Immediately upon the drawing of 5 cc. of blood, they are added to 100 cc. of citrated saline (prepared by adding 15 cc. of 1.5 per cent sodium citrate to 85 cc. of 0.85 per cent sodium chloride). The cell suspension is then transferred to 50 cc. graduated centrifuge tubes. The cells are separated by centrifuging at a moderate speed, after which the supernatant liquid is removed and the cells again suspended in saline, this being added to the 50 cc. mark in each of the tubes. The process is repeated three times so that in all, the cells receive four washings with saline. Finally the cells are suspended in saline, making a suspension 100 cc. of which contain the corpuscles from 5 cc. of the original blood. The actual number of corpuscles per unit volume may be

determined by count. The cells tend to settle out even on short standing. Accordingly when the suspension is used it should be mixed at frequent intervals.

Preparation of Acid Solutions.—In this work the acid solutions were prepared in 0.85 per cent sodium chloride. The stock solution was 0.1 N in strength, less concentrated solutions being prepared, as required, by dilution with physiological salt solution. A second set of solutions was prepared of the same acid concentrations but isotonic with 0.85 per cent sodium chloride. In the study of the effect of different concentrations of a given acid, the corresponding solutions were measured into small test-tubes of uniform diameter, 1.6 cc. being the volume used in each case. Determinations were usually made in triplicate. The pH of these solutions was determined potentiometrically, with a quinhydrone electrode. A volume of 16 cc. was usually found convenient. Since in the actual determination 0.4 cc. of the cell suspension is added to 1.6 cc. of the acid, the actual concentration of the acid at the beginning of hemolysis is the concentration of the acid after dilution with saline in the proportion: 4 parts of acid to 1 part of saline. Accordingly after an initial reading of the voltage was taken, 4 cc. of saline were added to the 16 cc. of the acid and a second reading taken, this reading being used in calculating the pH at the beginning of hemolysis.

Determination of Time Required for Complete Hemolysis.—All measurements were made at 25°. A large beaker nearly filled with water is fitted with a cover having a large hole through which a high form beaker is suspended. The cover should have three smaller holes, one for the thermoregulator, the second for a thermometer, and the third for a stirrer. The inside beaker likewise contains water and is fitted with a cover having three holes in a row, through which the test-tubes may be inserted, and two smaller holes, one for a thermometer and the second for a stirrer. A sheet of finely printed material (4 to 6 point type) is pasted around a part of the larger beaker, the letters facing the observer. In back of this screen a constant source of illumination should be placed.

The time required for hemolysis is determined with the aid of a stop-watch. Before a determination is made, the tubes containing the acid are brought to a temperature of 25° in a water bath.

When a determination is made one of the tubes is removed from the bath, 0.4 cc. of the red blood cell suspension is added rapidly, the contents mixed, and without delay the tube is suspended through one of the holes in the cover of the inside beaker. The procedure need not take more than 2 to 3 seconds; the stop-watch is pressed as the red blood cell suspension is added. The moment of complete hemolysis can be decided by comparison with a fully hemolyzed control tube placed in the bath. The time taken for a

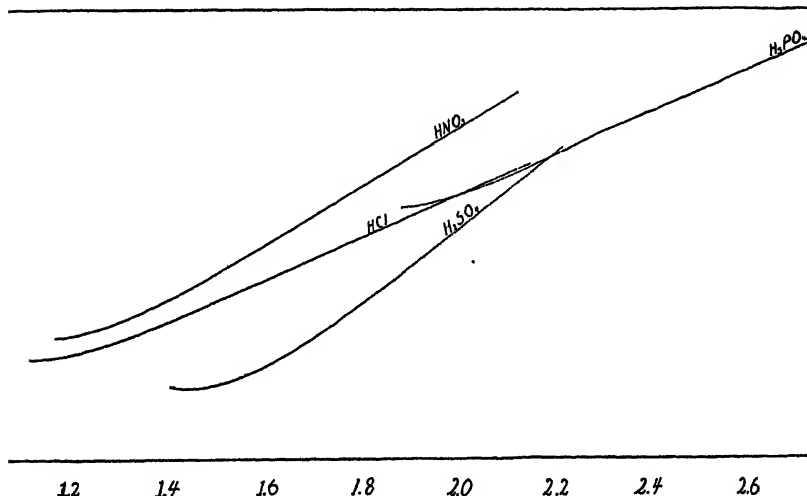


CHART I. Hemolytic action of inorganic acids. The curves show the relations between pH and rate of hemolysis of standard suspensions of dog erythrocytes.

particular dilution of acid to hemolyze 0.4 cc. of the blood suspension is then known. The method is very satisfactory, especially when hemolysis is relatively rapid.

Experimental Results.

In Chart I are represented the results obtained with hydrochloric, sulfuric, nitric, and phosphoric acids. These differ little in hemolytic action. The order of effectiveness in these experiments appeared to be: $\text{H}_3\text{PO}_4 > \text{H}_2\text{SO}_4 > \text{HCl} > \text{HNO}_3$.

Injury to the corpuscle rather than an osmotic effect seems to

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be the predominating factor in hemolysis by strong acids. In hemolysis by fatty acids, as will be shown later, an osmotic effect preceding hemolysis can be easily demonstrated. Jacobs (7) has shown that an osmotic effect is likewise responsible for the hemolytic action of the ammonium salts of the fatty acids.

TABLE I.
Effect of Tonicity of Acid Solutions on Hemolysis.

Concentration of acid.	0.1 N HCl.		0.05 N HCl.		0.1 N HNO ₃ .		0.05 N HNO ₃ .		0.1 N H ₂ SO ₄ .		0.05 N H ₂ SO ₄ .	
Tonicity.....	1.00	1.72*	1.00	1.36	1.00	1.70	1.00	1.35	1.00	1.40	1.00	1.20
Time, min.....	1.02	0.87	1.50	1.38	1.25	1.20	1.83	1.74	1.00	0.75	0.83	0.92
Concentration of acid.	0.1 N H ₃ PO ₄ .		0.05 N H ₃ PO ₄ .		0.1 N propionic.		0.05 N propionic.		0.1 N butyric.		0.05 N butyric.	
Tonicity.....	1.00	1.15	1.00	1.075	1.00	1.38	1.00	1.19	1.00	1.38	1.00	1.19
Time, min.....	2.60	2.45	2.95	2.90	1.25	2.70	3.67	4.33	1.05	2.10	3.67	4.78

* 1.72 × isotonic.

TABLE II.
Hemolysis of Standard Cell Suspensions. Comparison of Dog and Human Corpuscles.

Time required for hemolysis in minutes.

Acid.....	HCl				HNO ₃			
pH.....	1.09	1.35	1.72	2.10	1.12	1.42	1.82	2.22
Dog.....	0.97	1.55	2.42	3.20	1.10	1.75	3.07	3.97
Man.....	0.43	0.77	1.92	3.00	3.50(?)	1.16	2.25	3.43
Acid.....	H ₂ SO ₄				H ₃ PO ₄			
pH.....	1.22	1.51	1.89	2.15	1.90	2.11	2.44	2.84
Dog.....	0.79	0.94	2.12	3.50	2.58	3.02	3.83	4.50
Man.....	3.50(?)	0.90	1.67	3.08	1.78	2.80	4.20	5.75

Corpuscles suspended in a dilute solution of a fatty acid in physiological salt solution are apparently subjected to the following changes: the fatty acid penetrates the corpuscle, and, depending on the amount of acid, is partly or completely neutralized by the buffer substances in the cell; water penetrates into the cell to equalize the osmotic pressure on the two sides of the membrane; the corpuscle swells and eventually bursts. If this is the mech-

anism, small differences in osmotic pressure on the two sides of the membrane should have an important effect on the rate of hemolysis. This is the case for the fatty acids, but in the case of hemolysis by inorganic acids, a moderate increase in the osmotic pressure of the outside fluid seems to have no effect in delaying the rate of hemolysis. In fact, in some instances, the cells suspended in hypertonic solutions hemolyzed somewhat more readily. This is brought out by the data in Table I.

TABLE III.

Hemolysis of Standard Cell Suspensions by Inorganic Acids. Comparison of Resistance of Corpuscles from Normal and Anemic Dog.

Time required for hemolysis in minutes.

Acid	HCl				HNO ₃			
	1.12	1.44	1.83	2.15	1.18	1.47	1.87	2.22
pH.....								
Normal dog.....	1.02	1.50	2.30	3.00	1.25	1.83	2.94	3.50
Anemic " *.....	1.00	1.50	2.30	3.23	1.25	1.78	2.87	3.67
Acid	H ₂ SO ₄				H ₃ PO ₄			
	1.81	1.54	1.91	2.16	1.91	2.11	2.44	2.84
pH.....								
Normal dog	1.00	0.83	1.95	3.00	2.60	2.95	3.52	4.50
Anemic " *.....	1.05	0.88	2.08	3.22	2.33	2.88	3.70	4.67

* The red blood cell count of the anemic dog was 2.7 million per c. mm. as compared with a red blood cell count of 5.35 million for the normal animal. Accordingly, the cell suspension, in the case of the anemic dog, was prepared by use of twice the amount of blood taken normally. The number of corpuscles was therefore practically the same in the two suspensions.

Human corpuscles have a lower resistance to hydrochloric acid than dog corpuscles. For most concentrations within the effective range for rapid hemolysis this is also true for the other inorganic acids, as is indicated by the data in Table II.

It is interesting to observe that the resistance of corpuscles to inorganic acids does not change appreciably in experimental anemia produced by acetylphenylhydrazine, as is shown by the data in Table III. This result was quite unexpected in view of the known changes in the chemical composition of the red blood cell in this form of anemia.

TABLE IV.
Potentiometric Measurements during Hemolysis.

Series 11A. Dog corpuscles hemolyzed by hydrochloric acid.				Series 12B. Human corpuscles hemolyzed by nitric acid.			
0.1 N.				0.05 N.			
Time.	E.M.F.	0.05 N.		0.02 N.		0.01 N.	
min.	vols	Time.	E.M.F.	Time.	E.M.F.	Time.	E.M.F.
		min.	vols	min.	vols	min.	vols
0.23	0.3900	0.25	0.3735	0.16	0.3730	0.16	0.3525
0.50	0.3845	1.00	0.3680	0.33	0.3676	0.16	0.3450
1.00	0.3840	1.20	0.3660	0.58	0.3662	0.58	0.3445
2.00	0.3840	1.50	0.3640	1.00	0.3655	1.00	0.3430
		2.00	0.3640	1.25	0.3655	1.25	0.3425
				1.40		1.40	0.3415
				2.00		2.00	0.3415
							0.3175
Change.....	0.0060		0.0095		0.0075		0.0115
Equilibrium reached in: 0.25-0.5 min.	1.2 min.	1.5-2 min.		0.33-0.58 min.		1.25-1.4 min.	
Observed time for hemolysis: 0.97 min.	1.55 "	2.42 "		1.16 "		2.25 "	
						About 3 min.	
						3.43 "	

Permeability of the Red Blood Cell to Inorganic Acids.

The red blood corpuscle resembles other types of cells, such as the sea urchin egg, in being seriously injured in moderate concentrations of inorganic acids. It is likely that the change produced by the acid renders the red blood cell membrane freely permeable to most of the cell constituents and is accompanied or followed by the disintegration of the framework of the cell. When a red blood cell suspension is added to an acid solution the buffer constituents of the corpuscle neutralize a portion of the acid. The neutralization may be followed potentiometrically. It occurs promptly when hemolyzed cells are added but when the cells are intact the process requires a longer time, depending on

TABLE V.

Hemolysis of Dog Corpuscles in Varying Concentrations of Hydrochloric Acid.

0.1 N.		0.001 N.		0.0001 N.		Control in saline.	
Time.	Red blood cell count per c.mm.	Time.	Red blood cell count per c.mm.	Time.	Red blood cell count per c.mm.	Time.	Red blood cell count per c.mm.
0	80,000	0	80,000	0	80,000	0	80,000
0.25 min.	68,000	10 min.	78,000	1 hr.	80,000	1 hr.	80,000
2 "	400	1½ hrs.	100	20 hrs.	60,000	4 hrs.	72,000
10 "	400						
3½ hrs.	350						

the concentration of the acid and the rate of hemolysis. The point of equilibrium is however the same in either case. When hemolysis occurs rapidly there is very close correspondence between the time required for hemolysis, as determined by the visual method, and the time it takes the reaction between the acid and cell buffers to reach equilibrium. It cannot be argued, however, that the acid reacts with the buffer after liberation from the cell and that neutralization follows hemolysis. The two processes overlap each other. In relatively slow hemolytic reactions neutralization is complete before hemolysis. The data in Table IV illustrate this point.

The red blood cell remains intact in a 0.0001 N solution of hydrochloric acid almost as long as in salt solution (Table V). Pre-

sumably, even at this concentration the protein at the surface undergoes some change; the cell probably acquires a different electrical charge, but otherwise the toxicity effects are probably negligible. Even under these conditions acid diffuses into the cell and reacts with the cell buffers with considerable rapidity as shown by the data in Table VI. This is to be expected from what is known of the distribution of ions between the red blood corpuscle and its environment (Van Slyke, Wu, and McLean (8)).

Washed red blood corpuscles suspended in 0.001 *N* inorganic acid solution show practically no hemolysis during the first 10 minutes.

TABLE VI.

Neutralization of 0.0001 N HCl by Constituents of Red Blood Corpuscle.

Cell suspension.*		Control, hemolyzed cells.	
Time.	E.M.F.	Time.	E.M.F.
<i>min.</i>	<i>volts</i>	<i>min.</i>	<i>volts</i>
	0.1785		0.1785
0.16	0.1475	0.30	0.0910
0.42	0.1340	0.50	0.0820
0.75	0.1200	0.67	0.0755
1.00	0.1160	0.92	0.0720
2.00	0.1025	1.25	0.0675
3.00	0.0900	1.50	0.0660
4.00	0.0850	2.16	0.0630
6.00	0.0820	3.00	0.0630
13.00	0.0730	5.00	0.0630

* 4 cc. of a standard cell suspension were added to 16 cc. of acid. In the control, 4 cc. of hemolyzed corpuscles were added to 16 cc. of acid.

Yet, at the end of this interval the reaction between the acid and the cell buffers reaches completion. The curves in Chart II are based on the results obtained with the four inorganic acids studied in experiments in which both human and dog corpuscles were used. As in the preceding experiments the measurements were made at 25°, by means of a quinhydrone electrode. Quinhydrone does not seem to affect the rate of hemolysis. The acid (16 cc.) was measured into a beaker. The E.M.F. reading usually varied between 0.2700 and 0.2715 volts. The addition of 4 cc. of saline ordinarily produced a shift to about 0.2640 volts. This value was therefore taken to represent the initial hydrogen ion con-

centration of the solution. In the actual experiments 4 cc. of the standard cell suspension were added to the 16 cc. of acid, and readings were taken at short intervals. All the acids were ap-

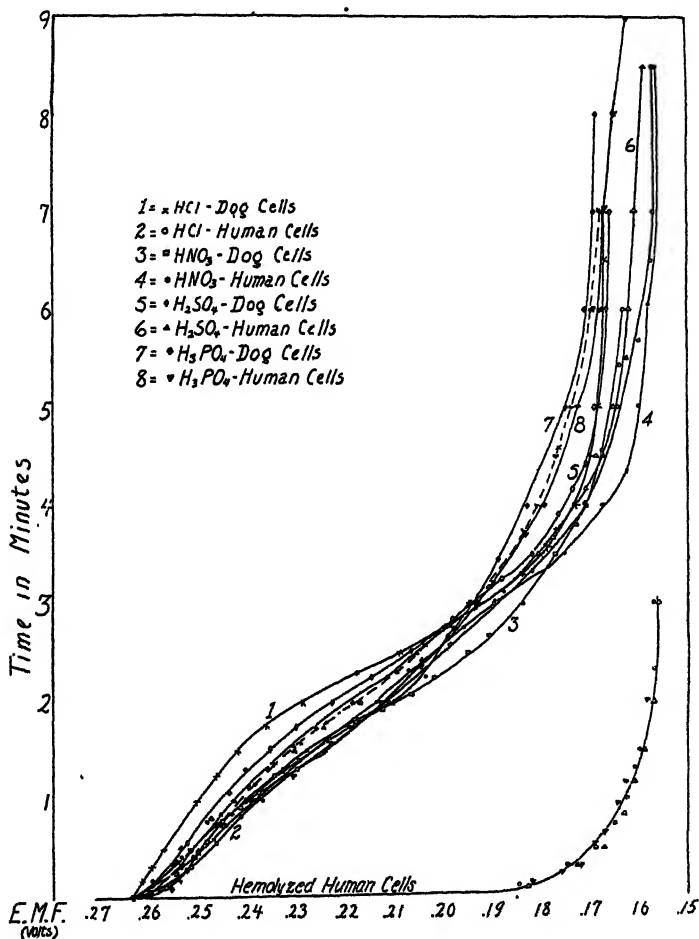


CHART II. The curves represent the neutralization of various inorganic acids by the constituents of dog and human corpuscles. Curve 1 is based on the results obtained with hydrochloric acid, with a freshly prepared suspension of dog corpuscles. The unnumbered broken line curve is based on results of a similar experiment with the same cell suspension after it had stood for 1 to 2 hours.

proximately 0.001 N in concentration, the phosphoric acid being 0.001 N with respect to the first hydrogen atom. The maximum shift in E.M.F. did not extend below 0.1560 volts in any of the experiments in this series. At 25°, this represents a pH of 5.01, at which point the dissociation of the second hydrogen atom of phosphoric acid is negligible.

It is to be seen from the curves in Chart II that the rates of penetration of the various acids into human and dog corpuscles did not differ greatly, although it is to be observed that in these experiments, hydrochloric and sulfuric acid penetrated the dog corpuscle somewhat more slowly than either phosphoric or nitric acid. The permeability of the washed corpuscle increases somewhat on standing. (Compare the broken and continuous lines for hydrochloric acid; the broken line is based on the results obtained with a cell suspension after it was allowed to stand for 1 to 2 hours.) The buffer content of washed human corpuscles was found to be greater than that of washed dog corpuscles. The same point of equilibrium was attained whether the reaction occurred between the acid and the constituents of the intact corpuscles or between the acid and hemolyzed corpuscles.

SUMMARY.

The order of effectiveness of the inorganic acids in producing hemolysis is: $\text{H}_3\text{PO}_4 > \text{H}_2\text{SO}_4 > \text{HCl} > \text{HNO}_3$.

In the concentrations necessary to produce rapid hemolysis (pH 1.0 to 2.8), injury to the cell membrane, rather than an osmotic effect, is the main factor responsible for the disintegration of the corpuscle by inorganic acids.

Human corpuscles have a somewhat lower resistance to inorganic acids than dog corpuscles.

The anemic and normal erythrocytes do not differ appreciably in their resistance to inorganic acids.

In hemolysis by acids, the reaction between the cell constituents and the acid is complete before hemolysis occurs. Inorganic acids react readily with the constituents of the intact corpuscle. This is partly due to the rapid diffusion, even from very dilute solutions, of inorganic acids, through the red blood cell membrane.

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LIPOID SOLUBILITY, PERMEABILITY AND HEMOLYTIC ACTION OF THE SATURATED FATTY ACIDS.*

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Hemolysis by fatty acids is closely related to the ability of these substances to penetrate the red blood cell membrane. Jacobs (1) has studied hemolysis produced by ammonium salts of the fatty acids and found that these owe their hemolytic effect to the permeability of the corpuscle to ammonia and the fatty acids, both entering the cell with relative ease even in the undissociated form. The accumulation of ammonium salts of the fatty acids inside the corpuscle leads to the osmotic effects which ultimately result in the disintegration of the cell.

The penetration of fatty acids into living tissues has been the subject of many excellent investigations (Loeb, Harvey, Crozier, Taylor, and others), but with the exception of the work of Jacobs with the ammonium salts and the essentially qualitative study of Fühner and Neubauer (2), the permeability of the erythrocyte to fatty acids and the hemolytic action of these substances have received little attention.

Loeb (3) studied the effect of different acids in the formation of artificial fertilization membranes in sea urchin eggs and found the order of effectiveness to be as follows: formic < acetic < propionic < butyric < caprylic < nonylic.

Harvey (4) investigated the penetration rates of different acids into the testis of the "prickly fish," which contains a purple water-soluble pigment sensitive to acids, and found that valeric acid penetrated much more rapidly than any of its lower homologues. Crozier (5) measured the penetration rates of various acids into

* A brief report of these observations was presented at the Ann Arbor meeting of the American Society of Biological Chemists, April, 1928.

the mantle fold of the mollusc, *Chromodoris zebra* and noted the following order of effectiveness: valeric > butyric > propionic > acetic. The effectiveness of formic acid was intermediate between valeric and butyric acids, but in view of the fact that Crozier compared the action of solutions that were 0.01 N in strength, this result is not surprising since the dissociation of formic acid is considerably greater than of its homologues. Crozier has also studied sensory activation by acids (6), using for this work the common earthworm, *Allobophora*. The effectiveness of the various fatty acids was in the order: acetic < propionic < butyric < valeric < formic < caproic < caprylic. A summary of related investigations is to be found in Jacobs' review in Cowdry's "General Cytology" (7).

More recently Taylor (8) determined the threshold concentrations necessary to produce a sour taste and found that for the lower members of the saturated fatty acid series higher concentrations are needed than for the higher members. He found that for solutions of equal pH a high degree of sourness is associated with a high penetration velocity of the undissociated acid or of the anion. It is to be seen therefore that in all the phenomena that have been studied in which primary penetration of the acid is a factor, a given fatty acid is invariably more active than its lower homologues and less active than its higher homologues.

In the present investigation a comparison has been made of the hemolytic action of the members of the saturated series of fatty acids, from formic to capric, including isobutyric, isovaleric, and isocaproic acids. A sufficient number of concentrations in isotonic saline were employed in order to establish for each acid the relationship between hydrogen ion concentration and the rate of hemolysis. In this work, parallel studies were made with dog and human corpuscles. The methods employed have been described in an earlier paper (9).

Even a condensed tabulation of the results that have been accumulated would require a considerable amount of space. The data for the dog corpuscle are therefore represented by means of the curves in Chart I. The data for human corpuscles will be considered separately. These curves bring out for the various acids their relative hemolytic power, and for the individual acids the relation between dilution and the rate of hemolysis. The

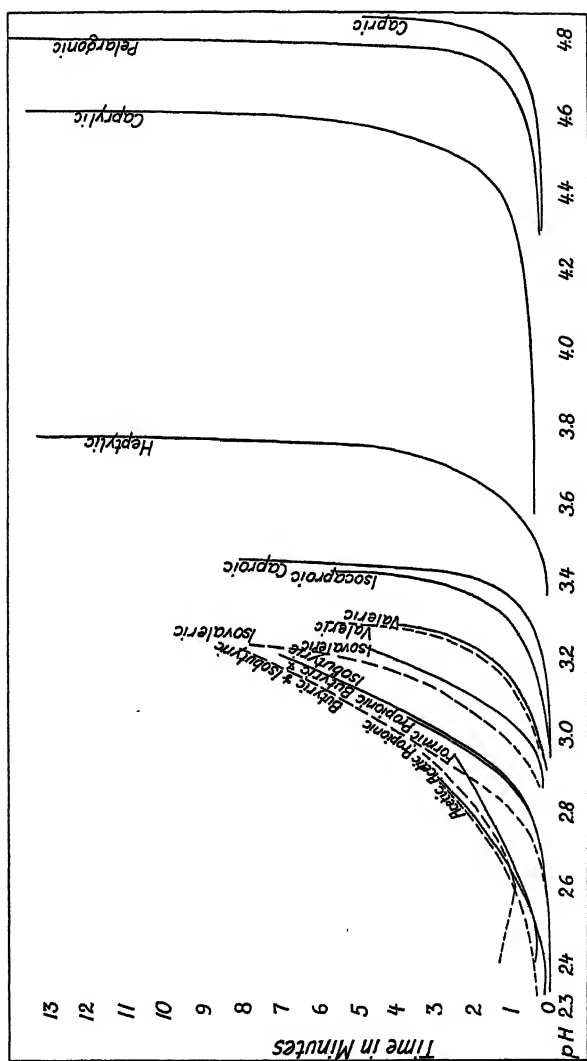


CHART I. Curves showing the relations between pH and the hemolytic action of the fatty acids. The determinations with capric acid were made at 35°. All other determinations were made at 25°.

close parallelism of the results upon which these curves are based and the results of those who have studied the permeability of various tissues to acids makes it difficult to escape the conclusion that the permeability of the fatty acids is intimately related to their hemolytic effect. Formic acid resembles in its action more nearly the inorganic acids than the remaining members of its series. The character of its dilution curve sets it apart from the other fatty acids and the fact that moderate increases in the osmotic pressure of the outside fluid produce relatively little effect on the rate of hemolysis leads one to suspect that primary penetration of the acid is not the main factor involved in the hemolysis by formic acid, but that this acid resembles the inorganic acids in injuring the corpuscles in some other way (9).

Two curves are given for acetic acid. The broken line is based on the results obtained with solutions prepared in isotonic salt solution. The hemolytic action of acetic acid is low. It requires a normal solution to hemolyze a standard cell suspension in about 0.3 minute. Now, a normal solution of acetic acid in water is approximately $4 \times$ isotonic, so that solutions of this acid, more concentrated than 0.25 N, even though prepared in water, are still hypertonic with respect to the cells. In view of the limited range of effectiveness of the acid, the concentrations above 0.25 N could not be excluded from the series, and the fact that these solutions were not isotonic is therefore to be borne in mind in interpreting the solid line for acetic acid, which is based on the results obtained with isotonic solutions of acetic acid of concentrations lower than 0.25 N and with solutions above this concentration that were unavoidably hypertonic. This applies also in the case of propionic acid, but for all the higher acids it was possible to adjust all the necessary concentrations to isotonicity. Two curves are given for propionic acid, the broken line being based on the data for the solutions prepared in saline and the solid line for the isotonic solutions. Two sets of curves are likewise given for butyric, isobutyric, valeric, and isovaleric acids in order to bring out the effect on the rate of hemolysis of small differences in the osmotic pressure of the outside fluid. The remaining curves are based on the results obtained with solutions prepared by dissolving the acid in 0.85 per cent sodium chloride. In the dilutions in which these acids were used, the addition of the acid to the physi-

ological salt solution was insufficient to produce any appreciable change in tonicity. A more detailed study of the relation of osmotic pressure to the rate of hemolysis is contemplated.

It will be observed that for a given pH, isovaleric acid is less effective than *n*-valeric acid and isocaproic acid is less effective than *n*-caproic acid in hemolyzing the corpuscles of the dog.

The higher fatty acids are very active hemolytic agents. These acids have a low solubility in water; the statements given in various handbooks that they are insoluble are, however, misleading. For example, a N/1500 solution of pelargonic acid is easily prepared. Pelargonic acid and its lower homologues are liquid at room temperature, and all the measurements with these acids were made at 25°. Capric acid, on the other hand, melts at 31°. The determinations with this acid were therefore made at 35°.

Under approximately uniform physiological conditions the properties of the red blood corpuscle of a given species are apparently such that experimental results of the type considered in this paper reproduce themselves from day to day with a uniformity that is very remarkable. Ponder (10) has observed this sort of consistency in his studies on saponin hemolysis. Corpuscles obtained from different normal individuals have approximately the same average resistance to saponin and the results which Ponder obtained with his own blood from time to time showed little variation. The present author has verified Ponder's assertions using his own blood, the data obtained being practically identical with those given by Ponder. Acids behave similarly in that over a long period of time there is little variation in the resistance of the corpuscles of a given animal towards a given acid. This does not mean, however, that the same mechanism is involved in the hemolysis by these two groups of substances; in fact the mechanisms are probably different.

The constancy in the behavior of the washed corpuscles to a given acid need not lead to the assumption that the permeability of the corpuscle is not subject to variation. The composition of the erythrocyte is far from being constant either physiologically or in a variety of pathological conditions. It is well known, for example, that the lipoids of the corpuscle are altered both qualitatively and quantitatively during fat absorption. Other changes

TABLE I.
*Data Showing Relative Resistance of Dog and Human Corpuscles to Varying Concentrations of Fatty Acids.**
 Time for hemolysis in minutes.

Acid.....	Formic.				Acetic.				Propionic.			Butyric.			Isobutyric.			Valeric.		
Normality....	0.1	0.05	0.02	0.01	1.0	0.5	0.25	0.1	0.5	0.25	0.1	0.5	0.25	0.1	0.5	0.25	0.1	0.05	0.02	
pH.....	2.39	2.52	2.73	2.93	2.32	2.47	2.63	2.85	2.53	2.70	2.92	2.49	2.67	2.86	2.52	2.68	2.89	3.06	3.27	
Dog.....	0.36	0.62	1.25	2.30	0.38	0.63	1.17	2.75	0.95	1.25	2.70	<0.05	0.33	1.80	<0.05	0.50	0.07	0.85	5.75	
Man.....	0.26	0.44	1.08	2.33	0.33	0.42	1.00	>4.00	1.22	3.42	>6.00	<0.05	5.75	>12.00	<0.05	7.00	0.11	2.08	>14	
Acid.....	Isovaleric.				Caproic.			Isocaproic.			Heptylic.			Caprylic.						
Normality....	0.1	0.05	0.02	0.1	0.05	0.02	0.01	0.1	0.05	0.02	0.01	0.02	0.01	0.005	0.002					
pH.....	2.86	3.03	3.22	2.92	3.05	3.24	3.42	2.89	3.03	3.22	3.42	3.36	3.43	3.61	3.78	4.33	4.46	4.53		
Dog.....	0.23	1.83	7.75	<0.02	0.05	0.33	3.17	Instantly.	0.05	0.75	5.60	0.09	0.21	1.37	17	1.16	4.00	7.45		
Man.....	0.94	>6.00	>13.0	0.02	0.06	2.00	17.0	0.02	0.07	3.15	>22.0	0.12	0.28	3.50	>35	2.58	12.5	>20		

* The solutions used in these determinations were prepared by dissolving the acid in physiological salt solution.

† The normality values are close approximations and represent the concentrations of acid before the addition of the standard cell suspensions.

‡ The pH values represent the actual hydrogen ion concentrations at the beginning of hemolysis and were obtained in separate determinations by diluting the acid solutions with saline in the proportion of 1 cc. of saline to 4 cc. of acid. In determining the time required for complete hemolysis, 0.4 cc. of the standard corpuscle suspension is added to 1.6 cc. of the acid solution.

occur in experimental anemia. It is conceivable that changes such as these in the composition of the cell are likely to be associated with variations in the rate of penetration of various substances into the corpuscle.

Variations in permeability of acids would alter somewhat the position and shape of the dilution curves of the type given in Chart I, but despite such variations, the order of effectiveness of the different members of the series as represented by these curves will probably be found to hold under a large variety of conditions for all mammalian corpuscles.

A comparison of hemolysis of human and dog corpuscles by fatty acids has brought out certain distinctions. It will be recalled that human corpuscles are somewhat less resistant to inorganic acids than dog corpuscles. This is also true for formic and acetic acids. The resistance to propionic acid, on the other hand, is somewhat greater in the case of human cells, and for all the higher fatty acids the human corpuscles show a distinctly greater resistance to hemolysis than dog corpuscles. This is brought out by the data outlined in Table I.

The greater resistance of the human corpuscles appears to be related to their higher content of buffer substances. In measuring the shift in potential of the acid solutions during hemolysis, the washed human corpuscles invariably neutralized a greater amount of acid than dog corpuscles. In hemolysis with the lower fatty acids this effect is bound to be of relatively little importance because of the high concentration of acid needed to bring about hemolysis and of the relatively small proportion of this acid neutralized by the constituents of the cells. Yet, even in a 0.1 N solution of acetic acid the human corpuscles exhibit greater resistance than dog corpuscles. In the case of the higher fatty acids where lower concentrations suffice to bring about hemolysis, the difference in the neutralizing capacity of the constituents of the two types of cells may be sufficient to reduce appreciably the amount of available acid and lead to a reduction in the rate of hemolysis. It is to be seen from the data in Table I, as well as from the shape of the curves in Chart I, that a small decrease in the concentration of acid may result in a very pronounced retardation of hemolysis. For example, in the case of caproic acid, at pH 3.4, hemolysis of the standard cell suspension

occurred in about 2 minutes, whereas when the initial pH was 3.45, complete hemolysis did not occur until after an interval of 8 minutes.

The data in Table II have been selected at random from the results obtained in the measurement of hemolysis by means of the potentiometer. The initial pH values of the acid solutions are given, as well as the values at the end of the reaction between the acid and cell constituents. While the differences in the pH shift between the human and dog corpuscles for the various acids is not great, nevertheless differences of like magnitude have a

TABLE II.

*Shift in pH Due to Neutralization of Various Acids by Constituents of Dog and Human Corpuscles.**

Acid.....}	Acetic.	Pro-pionic.	Butyric.	Valeric.	Iso-valeric.	Caprylic.
Approximate normality....	0.5	0.5	0.1	0.1	0.02	0.001
Dog.						
Initial pH.....	2.40	2.50	2.77	2.80	3.12	4.30
Final pH.....	2.58	2.73	3.12	3.11	3.62	5.02
Man.						
Initial pH.....	2.40	2.50	2.77	2.80	3.12	4.28
Final pH.....	2.63	2.80	3.18	3.17	3.73	5.20

* To 16 cc. of acid, 4 cc. of the standard red blood cell suspension were added.

considerable effect on the rate of hemolysis, especially for the higher fatty acids, as may be seen by projecting these values on certain portions of the curves given in Chart I.

Mechanism of Penetration of Fatty Acids into Red Blood Corpuscle.

In his study of cell permeability, Harvey (4) did not obtain clear cut evidence either to support or contradict Overton's well known lipid solubility theory. Harvey states, however, that lipid solubility, or capillary activity, for the two run more or less parallel, constitutes one of the factors upon which the penetration of acids depends.

Taylor's work on taste (8) has led him to conclude that sourness is not purely a function of the stoichiometric acid concentration, nor of the hydrogen ion concentration. In attempting to find an

explanation for his results he has assumed that the production of a given degree of sourness is due to the establishment within the cells of the taste bud of a definite hydrogen ion concentration and from his experimental results he has calculated the concentration gradients of equally sour acids. He recalculated similarly the taste data of Paul and Bohnen (see Paul (11)), and from the data of Crozier (5, 6) computed the concentration gradients of acids penetrating *Chromodoris* tissue, as well as the

TABLE III.

Concentration Gradients of Acids Penetrating the Red Blood Corpuscle and Producing Hemolysis of a Standard Cell Suspension in 1 Minute.

Acid.	pH	Total acid concentration.	H ⁺ ion concentration.	Concentration of undissociated acid outside. (a)	Relative concentration of undissociated acid inside. (b)	Ratio (a): (b).	Ratio (a): (b) when acetic acid = 1.
		$\times 10^{-2}M$	$\times 10^{-3}M$	$\times 10^{-3}M$			
Formic.....	2.67	23.5	2.14	21.4	1.00	21.4	
Acetic.....	2.64	286	2.3	284	11.5	24.7	1.00
Propionic.....	2.86	141	1.4	140	15.2	9.2	0.372
Isobutyric.....	2.87	122	1.35	121	14.3	8.46	0.342
Butyric.....	2.87	124	1.35	123	14.5	8.48	0.344
Isovaleric.....	3.03	51.9	0.93	51	12.6	4.05	0.164
Valeric.....	3.14	34.1	0.73	33.4	13.4	2.50	0.101
Isocaproic.....	3.28	19.2	0.53	18.7	14.3	1.81	0.053
Caproic.....	3.35	14.4	0.45	13.9	14.7	0.95	0.038
Heptylic.....	3.58	4.9	0.26	4.64	14.7	0.315	0.0128
Caprylic.....	4.33	0.2	0.047	0.153	14.9	0.0103	0.0004
Pelargonic.....	4.67	0.06	0.021	0.04	19.4	0.0021	0.000084

concentration gradients causing retraction in the earthworm. The resulting values, not only showed agreement with each other, but resembled Freundlich's data (12) on the relative concentrations of undissociated fatty acids (formic, acetic, propionic, and butyric) which are necessary in order that charcoal may adsorb the same quantity of acid in each case. Accordingly, Taylor suggests that the fatty acids are taken into tissues by an adsorption process.

It is probably unnecessary to postulate an adsorption mechanism, for the results of acid penetration into tissues can be more

fully correlated on the basis of solubility data which are outlined in this paper. In order to compare our results on hemolysis with the data that have been obtained by others in a variety of biological phenomena involving the penetration of acids into tissues, the relative concentration gradients of undissociated acids across the red blood cell membrane, causing hemolysis (a) in 1 minute, and (b) in 5 minutes, have been determined by the methods suggested in Taylor's paper. The results are outlined in Tables III and IV. The relative concentrations of undissociated acid

TABLE IV.

Concentration Gradients of Acids Penetrating the Red Blood Corpuscle and Producing Hemolysis of Standard Cell Suspension in 5 Minutes.

Acid.	pH	Total acid concentration.	H ⁺ ion concentration.	Concentration of undissociated acid outside. (a)	Relative concentration of undissociated acid inside. (b)	Ratio (a):(b).	Ratio (a):(b) when butyric acid = 1.
		$\times 10^{-3}M$	$\times 10^{-3}M$	$\times 10^{-3}M$			
Butyric.....	3.12	39.9	0.76	39.1	14.5	2.70	1.00
Isobutyric.....	3.12	39.3	0.76	38.5	14.3	2.70	1.00
Isovaleric... ..	3.23	21.1	0.59	20.5	12.6	1.63	0.60
Valeric.. ..	3.30	16.2	0.50	15.7	13.4	1.17	0.43
Isocaproic.....	3.42	10.0	0.38	9.6	14.3	0.67	0.25
Caproic.....	3.44	9.3	0.36	8.9	14.7	0.60	0.22
Heptylic.....	3.75	2.3	0.175	2.1	14.7	0.143	0.053
Caprylic.....	4.58	0.073	0.026	0.047	14.9	0.0032	0.0012
Pelargonic.....	4.78	0.043	0.017	0.026	19.4	0.0013	0.0005

inside the cell are based on the dissociation constants of the fatty acids, as given in the "Landolt-Börnstein Physikalisch-chemische Tabellen." The present writer has been unable to find data for the dissociation constant of capric acid; the results for this acid are therefore omitted from the tables.

The ratios given in the last column of Tables III and IV may be taken to represent the relative gradients or driving forces which are necessary to cause the various fatty acids to penetrate into the corpuscles of the dog to a comparable degree. When allowance is made for the higher concentrations of acid necessary to

bring about hemolysis than are required to produce a sour taste or to cause sensory activation as in Crozier's experiments (6), the relations indicated by the data in Tables III and IV nevertheless bear a close resemblance to the relations that have been shown to exist in other biological phenomena which depend upon the primary penetration of the acids into tissues. That the primary penetration of the fatty acids into the erythrocyte depends on their solubility in the lipoids of the cell will now be considered.

It is generally known that the lipid solubility of the members of the saturated series of fatty acids increases according to the size of the molecule. However, adequate data bearing on the distribution of the fatty acids between water and fatty substances seem to be lacking. In his paper on the permeability of cells to acids, Harvey (4) includes certain data obtained from Wasteneys on the partition coefficients between water and olive oil of several of the fatty acids in 0.01 N concentration.¹ In view of the possible relationship between the penetrability of the fatty acids into the corpuscle and lipid solubility and because of the lack of solubility data, it seemed desirable to determine the partition coefficients of the fatty acids between water and olive oil. (The same results were obtained with aqueous solutions as with solutions of the fatty acids in saline.)

The solubility of the fatty acids was determined at 23°. 100 cc. portions of the various acids of known concentration (N, 0.1 N, 0.01 N, 0.001 N) were transferred to flasks. After the removal of 25 cc. for the preliminary titration with alkali, 5 cc. of olive oil (Squibb) were added to the remainder; the flasks were tightly stoppered and the contents shaken in a mechanical shaking device for 1½ hours. As a rule the distribution was found to reach equilibrium within 30 minutes. After the oil was allowed to separate, a second 25 cc. portion of the solution was titrated. The oil was then transferred to narrow test-tubes and set aside for 24 hours or longer to insure complete separation of the oily layer. Accurately measured portions of the oil were then dissolved in neutral alcohol and titrated with standard alkali. Suitable control analyses were made for the acid content of the olive oil. Excellent agreement was obtained in nearly all of the experiments

¹ Except for Harvey's citation, this work has not been published, according to a personal communication from Dr. H. Wasteneys.

TABLE V.
Solubility of Fatty Acids in Olive Oil.

Acid.	Acid lost from 75 cc. water and recovered in 5 cc. oil.	Acid in 1 cc. water at equilib- rium.	Acid in 1 cc. oil at equilib- rium.	Oil:water distribu- tion ratio.	Water:oil distribu- tion ratio.
		C ₁	C ₂	C ₂ :C ₁	C ₁ :C ₂
1.0 N.					
	cc.	cc.	cc.		Formic acid = 1.
Formic.....	0.11	0.9985	0.022	0.022	1.00
Acetic.....	0.15	0.998	0.03	0.030	0.73 }
Propionic.....	1.05	0.986	0.21	0.213	0.104
Butyric.....	7.5	0.900	1.5	1.67	0.013
0.1 N.					
					Formic acid = 1.
Formic.....	0.025	0.9997	0.005	0.005	1.00
Acetic.....	0.15	0.998	0.03	0.03	0.165
Propionic.....	0.7	0.991	0.14	0.141	0.035
Isobutyric.....	3.1	0.959	0.62	0.645	0.0077
Butyric.....	3.1	0.959	0.62	0.645	0.0077
Isovaleric.....	11.0	0.853	2.2	2.58	0.0019
Valeric.....	17.2	0.77	3.45	4.48	0.0011
0.01 N.					
					Propionic acid = 1.
Propionic.....	0.75	0.99	0.15	0.1515	1.00
Isobutyric.....	2.15	0.972	0.43	0.443	0.342
Butyric.....	2.15	0.972	0.43	0.443	0.342
Isovaleric.....	8.5	0.887	1.7	1.92	0.079
Valeric.....	11.5	0.847	2.2	2.60	0.058
Isocaproic.....	26.0	0.653	5.2	7.96	0.019
Caproic.....	29.0	0.613	5.8	9.47	0.016
Heptylic.....	57.5	0.233	11.5	49.3	0.003
0.001 N.					
					Butyric acid = 1.
Propionic.....	0	1.00	0		
Isobutyric.....	1.5	0.98	0.3	0.306	1.00
Butyric.....	1.5	0.98	0.3	0.306	1.00
Isovaleric.....	7.5	0.90	1.5	1.67	0.184
Valeric.....	9.0	0.88	1.8	2.05	0.150
Isocaproic.....	16.5	0.78	3.3	4.23	0.072
Caproic.....	22.5	0.70	4.5	6.43	0.048
Heptylic.....	40.0	0.47	8.0	17.0	0.018
Caprylic.....	51.0	0.32	10.2	31.9	0.0095

between the amount of fatty acid lost from the aqueous layer and the amount of fatty acid recovered in the oil. The results, which are based on several series of determinations, are outlined in Table V.

Owing to the low solubility of pelargonic acid, concentrations lower than 0.001 N were employed. With such dilutions, the procedure of titrating the acid solutions before and after shaking with oil was subject to considerable error. The more accurate

TABLE VI.
Solubility of Fatty Acids in Olive Oil.

Acid:	pH shift.		Total acid concentration.		Acid lost from 75 cc. water.	Acid in 1 cc. water at equilibrium. C_1	Acid in 1 cc. oil at equilibrium. C_2	Oil: water distribution ratio. $C_2:C_1$	Water: oil distribution ratio. $C_1:C_2$
	Initial pH.	pH at equilibrium.	Initial $\times 10^{-4}M$.	At equilibrium $\times 10^{-4}M$.					
					Cc. 0.000407 N.				
Isocaproic.....	4.15	4.23	0.407	0.291	21.5	0.713	4.30	6.04	0.166
					Cc. 0.000375 N.				
Heptylic.....	4.17	4.35	0.375	0.184	38.2	0.49	7.64	15.6	0.064
					Cc. 0.000443 N.				
Caprylic.....	4.14	4.64	0.443	0.060	64.85	0.135	12.97	96.1	0.014
					Cc. 0.000363 N.				
Pelargonic... ..	4.24	5.02	0.363	0.00934	73.125	0.025	14.625	585	0.0017

procedure was therefore adopted of measuring the change in hydrogen ion concentration. Determinations were made with solutions of isocaproic, heptylic, caprylic, and pelargonic acids of approximately the same concentration. The results are given in Table VI.

An examination of the distribution ratios given in Tables V and VI and comparison of these data with the relative concentration gradients given in Tables III and IV, as well as with the other

data contained in this paper, lead to the conviction that lipoid solubility is an important factor in determining the rate of penetration of the fatty acids into the erythrocyte and the order of effectiveness of the various members of the series in producing hemolysis.

Since the data outlined in this paper are not difficult to correlate, a detailed discussion seems unnecessary, but for the purpose of illustrating how well the results agree, the following example will be considered. It is to be noted that the distribution ratios for butyric and isobutyric acid are identical for the concentrations included in Table V. The hemolysis curves (Chart I) are likewise identical, and accordingly the relative concentration gradients for these acids are the same (Table III and IV). On the other hand, the position occupied by isovaleric acid in the order of effectiveness as a hemolytic agent is intermediate between butyric and *n*-valeric, and isocaproic acid occupies a position intermediate between valeric and *n*-caproic acids. These are precisely the positions occupied by the oil : water (and water : oil) distribution ratios of isovaleric and isocaproic acids.

Incidentally, it is to be observed that the oil : water distribution ratios $\frac{C_2}{C_1}$ for the members of the series above propionic acid increase with increasing concentration of the acids. This indicates that the degree of molecular association of the fatty acids is greater in olive oil than in water. It is likely that this is also true for formic, acetic, and propionic acids, but in view of the low solubility of these in oil, the methods employed in this work were inadequate to bring this out with certainty.

SUMMARY.

The order of effectiveness of the fatty acids in penetrating the red blood corpuscle and producing hemolysis is: acetic < propionic < butyric = isobutyric < isovaleric < valeric < isocaproic < caproic < heptylic < caprylic < pelargonic < capric.

The relations between pH and hemolytic action have been determined for each of these fatty acids and the results represented by means of curves.

Increasing the osmotic concentration of the outside fluid retards

hemolysis by fatty acids. This shows that osmotic effects are involved in the process.

Formic acid resembles the inorganic acids in its hemolytic action.

Human corpuscles are more resistant to hemolysis by fatty acids than dog corpuscles. This is apparently due to the greater content of buffer substances in washed human corpuscles than in washed dog corpuscles and the neutralization of a greater amount of acid.

Determinations have been made of the relative concentration gradients of the acids, penetrating the corpuscle and producing hemolysis. The relations which are shown to exist in hemolysis resemble closely the relations that have been observed by other workers in a variety of phenomena in plant and animal tissues involving the primary penetration of the acids.

Determinations have been made of the distribution of the fatty acids between water and olive oil. A close parallelism exists between lipoid solubility and the effectiveness of the fatty acids in producing hemolysis.

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RICKETS IN RATS.

VII. METABOLISM OF CALCIUM AND PHOSPHORUS OF RATS FED UPON NON-RICKETOGENIC DIETS.*

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To evaluate the metabolism of calcium and phosphorus, in our previous experiments animals on various deficient diets have been compared to "normals." The standards adopted for calcium and phosphorus metabolism were those of Sherman for analyses of rats, of the Wistar Institute for bone analyses, and of the Lister Institute for metabolism studies. As their diets differed more or less widely from those we used, it seemed advisable to modify our diets toward normal by the addition of salts and vitamins, only one alteration being made at a time. In this way, we have been able to estimate, stepwise, the effect of the deficiency of the diet.

What constitutes normality is more or less a philosophical question. One is reminded of the ophthalmologist who said that in long years of experience he had seen but one normal eye. The usual meaning of the term is the median or mode of those individuals showing no known abnormalities. Chittenden and Mendel (1) emphasize that the average depends upon what is considered a proper diet. Experience with the albino rat has shown that our concept of the normal is changing.

Experiments of the Wistar Institute over a long period have resulted in gradual improvement of the average growth. Weight curves over a period of years have shown increasingly good animals (Osborne and Mendel (2)). Whether these animals represent

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accelerated early growth or actual increased size at maturity has not been determined. So far as known, neither of the laboratories mentioned has made special effort to select those animals for breeding which showed the best growth. Any conscious selection was to weed out the unfit. With regard to the nutrition of the animals, diets of the previous generation and especially of the mothers during pregnancy and lactation affect markedly the growth of the young. Even when these factors are kept constant, longer experience in handling animals gives progressively better results. Other standards—relative length and proportion of the body, the growth of special parts or organs—give results that differ from those of weight alone (3). In this study normality was judged by gross weight increases, and by histological appearance and ash content of the bones.

Our effort has been not to obtain the best possible growth, but so to adjust conditions that constant and reproducible experimental material was secured. We have tried to make animals ricketic under exactly the same conditions so that the curative effects of various procedures could be studied upon a standard ricketic animal. The quality or amount of protein, fat, or carbohydrate, has not been changed. Attention has been directed to slight alterations in the mineral composition of the diet and to the effect of vitamins. The diet, of course, should be considered as a whole: change in one constituent affects the relations of all the others. A diet which may be satisfactory with a given calcium content may be unsatisfactory if the protein, fat, vitamins, or any of the mineral elements is changed.

Plan of Experiment.—Rats reared in our laboratory were kept with their mothers until 21 days old. Mothers and young had access to the Sherman Diet B, which consists of two-thirds whole wheat and one-third whole milk plus 1.33 per cent of NaCl. Then the young were taken from the mother and continued on the same diet until 28 days old. They were then placed in metabolism cages of the Hopkins type and fed the diet to be tested. The animals were studied for the subsequent 35 days. In addition to general behavior and growth, the following were considered: blood serum analyses for calcium and phosphorus, histologic examination of the bones, analysis of the ash content of the fat-free bones, and metabolism of calcium and phosphorus.

Care of the animals, composition of the diets, and the analyses, were as previously described (4). Three to six animals in separate metabolism cages were used for each experiment, with approximately an equal number of males and females. The diet used to produce rickets was that of Steenbock and Black, Ration 2965 (5), which consists of 76 per cent yellow corn, 20 per cent gluten flour, 3 per cent CaCO_3 , and 1 per cent NaCl . To 100 gm. of this mixture were added 10 gm. of lard. The calcium by analysis was 1.08 per cent and the phosphorus 0.254 per cent. The ratio of $\text{Ca:P} = 4.1$. 100 gm. of the ration are equivalent to 530 cc. of 0.1 N alkali. This was Diet A. It was altered by the addition of enough $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ to make the phosphorus

TABLE I.
Calcium and Phosphorus in Blood Serum of Rats Fed Modifications of Steenbock and Black's Ration 2965, for 35 Days.

Calcium.	Phosphorus.	Histological examination.	Diet.
<i>mg. per cent</i>	<i>mg. per cent</i>		
10.0	2.6	Marked rickets.	Steenbock's diet + 10 per cent lard (Diet A).
10.4	4.2	Normal.	Diet B ($\text{Ca:P} = 2.0$).
		"	Diet C (4.0 per cent butter).
11.4	8.0	"	Diet D (2 per cent cod liver oil).
12.9	6.9	"	Diet E (irradiated corn + 2 per cent cod liver oil).

0.54 per cent. The ratio of Ca:P was approximately 2.0. This ration per 100 gm. is equivalent to 392 cc. of 0.1 N alkali, and was called Diet B. Diet C was made from Diet B; 4 gm. of lard were replaced by 4 gm. of butter fat (tested as to its vitamin A content). Diet D was made from Diet B; 2 gm. of lard were replaced by 2 gm. of cod liver oil (tested for vitamins A and D). Diet E was made from Diet D; the corn was irradiated for 20 minutes at a distance of 30 inches with a Hanovia Alpine Lamp known to be active. The cod liver oil was fed separately.

Results.

Blood Serum Values.—Values of the blood serum for calcium and phosphorus, when the animals had been fed on the experi-

mental diets for 35 days, are shown in Table I. Diet A resulted in the low phosphorus characteristically associated with this rickets-producing diet. With the increase in phosphate content of the diet (Diet B), the phosphorus rose to a low normal value. Further addition of vitamins (Diets D and E) resulted in a further increase of phosphorus and also of calcium.

Histological Examination of Bones.—Changes in the salt composition of the diet were sufficient to produce a bone which on section was practically normal (Table I). Additional improvements in the diet were without further visible effect.

Bone Analyses.—Values of the bone analyses by the technique of Chick and Roscoe (6) are shown in Table II. The effect of the various diets is clear. The additions of an amount of phos-

TABLE II.
Analyses of Femora of Rats.

Six femora.

Diet.	Ca:P.	Age.	Weight.				Ash, per cent of fat-free.
			Wet.	Dry.	Fat-free.	Ash.	
		days	gm.	gm.	gm.	gm.	
A	6.0	61	1.56	0.671	0.540	0.168	31.6
B	2.25	65	1.64	1.035	0.881	0.530	60.1
C	1.9	65	1.45	0.876	0.760	0.358	47.0
D	2.7	65	2.37	1.321	1.202	0.725	60.4
E	2.40	65	2.65	1.463	1.334	0.766	57.6

phorus sufficient to make the ratio of Ca:P = 2.0, or optimal, resulted in bones normal as to percentage composition of the ash. When butter fat was fed for 4 weeks, the bone obtained was slightly less good. Cod liver oil and cod liver oil plus irradiated diet did not increase the per cent of ash in the bone above normal, but gave a larger bone. With the exception of the butter fat diet, increase in the size of the bones is progressive from Diet A to Diet E. The maximal effect is obtained not, as might be expected, with cod liver oil, but with cod liver oil supplemented with irradiated corn.

Metabolism.

Weights of Animals.—Although the rats had free access to the food at all times the different groups grew at different rates.

The individuals in each group were so nearly alike that only the averages are shown in Table III. On the rickets-producing diet they grew poorly, actually losing weight for the last 2 weeks. The rate of growth improved progressively from the slowest, when the rats were given Diet A, to the fastest when they received

TABLE III.

Gain in Weight, Food Intakes, and Feces of Rats Fed Ricketogenic and Non-Ricketogenic Diets.

Diet.	Initial weight.	Final weight.	Period No.					Average.
			1	2	3	4	5	
Gain per wk. per rat.								
	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
A	48	63	7.0	7.0	7.0	-3.0	-2.0	3.0
B	52	72	2.3	4.0	6.7	2.8	5.1	4.2
C	43	63	*	6.7	3.7	5.1	1.1	4.1
D	60	105	8.8	12.5	11.1	6.1	6.0	8.9
E	56	128	11.7	16.7	15.0	14.0	14.1	14.3
Net food per wk.								
A								30.8
B			32.9	35.0	36.4	33.6	33.6	34.3.
C			*	35.0	32.2	30.8	25.9	30.8
D			53.9	56.0	53.9	60.9	41.3	53.2
E			52.5	66.5	64.4	74.2	71.4	65.8
Feces; dry weight per wk.								
A								3.6
B			3.58	3.59	3.57	3.43	3.96	3.62
C			*	2.71	3.69	3.54	3.43	3.34
D			5.24	5.65	6.26	5.94	5.02	5.62
E			5.32	7.66	6.16	7.70	7.92	6.95

* Group C was fed on Diet B for the 1st week.

Diet E. The maximal effect was obtained not when cod liver oil was added to a diet containing a good salt mixture, but when this diet was further improved by irradiation. The combined addition of light and cod liver oil to the diet resulted in normal growth. With Diet A the animals gained only for the first 3 weeks. With Diet B there was moderate growth throughout the

TABLE IV.

Metabolism of Calcium and Phosphorus.

Figures are in terms of one rat per week.

Diet.	Period.	Calcium.							Phosphorus.						
		Intake.	Urine.	Stool.	Spill.	Total output.	Balance.	Per cent.	Intake.	Urine.	Stool.	Spill.	Total output.	Balance.	Per cent.
	<i>wks.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>
B	I	365	11	178	+6	205	+160	44	163	11	87		98	+65	40
	II	392	13	218	+9	240	+152	39	175	7	107	+2	116	+59	34
	III	393	19	218	+6	243	+150	39	195	11	110		121	+74	38
	IV	365	11.6	221	+11	248	+117	32	181	7	117	+4	128	+53	29
	V	362	18	267	+5	290	+72	20	179	7	143	+2	152	+27	15
Average...		375	14.7	251		245	130	35	179	8	113		123	56	31
C	II	394	15	159	+10	184	+210	53	205	11	73	-3	81	+124	60
	III	362	20	213	+10	243	+119	33	188	17	103	-2	118	+70	37
	IV	344	18	215	+6	239	+105	31	179	13	109	-1	121	+58	32
	V	291	21	188	+2	211	+80	27	152	14	95		109	+43	28
Average...		348	18	194		219	129	37	181	14	95		107	74	41
D	I	558	49	256	+75	380	+178	32	209	6	88	+2	96	+113	55
	II	585	58	316	+47	421	+164	28	218	7	126	+5	138	+80	38
	III	564	76	346	+36	458	+106	19	210	4	154	+1	159	+51	24
	IV	631	55	317	+16	388	+245	39	236	3	131	+2	136	+100	41
	V	433	48	291	+16	355	+88	20	162	3	115	+1	119	+43	25
Average...		554	57	363		400	156	28	207	5	123		130	77	27
E	I	560	64	275	+30	369	+191	34	235	5	100	-2	103	+132	56
	II	710	93	391	+84	568	+142	20	299	4	153	+5	162	+137	46
	III	693	61	333	+42	436	+257	37	291	5	137	-1	131	+150	52
	IV	850	104	434	+65	603	+247	29	358	7	170	-12	165	+193	55
	V	749	103	457	+65	625	+124	17	282	5	169	+3	177	+105	37
Average...		712	85	378		520	192	27	293	5	146		149	144	49

5 weeks. When butter was added (Diet C) moderate growth took place for only 1 week. At the end of the period the animals were definitely failing. Cod liver oil (Diet D) gave its best results for the first 3 weeks. Diet E containing cod liver oil plus irradiated corn, not only gave the best growth, but the rate of growth was maintained throughout the experimental period.

Food Intakes.—Consumption of the diets containing added phosphate and butter was similar to that previously reported for the rickets-producing diet, 30 to 40 gm. per week. When cod liver oil was added more food was consumed; when irradiation was combined with cod liver oil still more food was eaten. Animals on the basal diet ate approximately one-half the amount

TABLE V.
Distribution of Ingested Calcium and Phosphorus.

Diet.	Calcium.			Phosphorus.		
	Urine.	Feces.	Retention.	Urine.	Feces.	Retention.
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
A	17	57	25	4	69	27
B	4	67	35	4	63	31
C	5	52	37	8	51	41
D	10	55	28	3	60	37
E	12	53	22	2	49	49

consumed by those fed upon the irradiated diet plus cod liver oil. The data are given in Table III.

Weight of Feces.—The dried feces increased progressively in weight from Diet A to Diet E except for the low values on the diet containing butter (Table III). These values correlate exactly with the gains in weights of the animals and the food consumption. They demonstrate, further, approximately the same degree of utilization; for in all cases, the dry feces equal 10.5 to 11.5 per cent of the weight of the food eaten.

Metabolism of Calcium and Phosphorus. Paths of Excretion.—The data for the intakes and outputs of calcium and phosphorus are given in Tables IV and V. Compared with the rickets-producing diet (7), Diet B, containing added phosphate, caused less calcium to be excreted in the urine. Calcium excretion in the feces remained approximately the same. The phosphorus ex-

cretion, on twice the intake, was approximately doubled in both urine and feces. When the diet was further improved (Diets C, D, and E) both urinary and fecal calcium increased. The phosphorus in the feces was increased slightly and that in the urine was constant or diminished. Because the intakes of both elements were progressively increased with the last three diets, such a comparison may be misleading. The relations are perhaps more clearly brought out when the excretions are compared with the intakes. 4 to 12 per cent of the calcium was in the urine, 52 to 67 per cent in the feces. 3 to 14 per cent of the phosphorus was in the urine and 49 to 67 per cent was in the feces. Table V reveals that the relative percentage of calcium and of phosphorus excreted by the urine and feces is roughly the same for all the diets.

Balances of Calcium and Phosphorus.—The positive balances increased progressively from Diet A to Diet E (Table IV). Each of the diets with added phosphorus resulted in a retention not only of more phosphorus but also more calcium. As the diets were improved a smaller percentage of calcium was retained and greater percentage of phosphorus, so that the ratios of retention fell progressively from 4.0 on the rickets-producing diet to 1.33 on the diet containing both irradiated corn and cod liver oil. Difference in the actual amounts of calcium and phosphorus retained depends primarily upon the food intakes.

DISCUSSION.

Boas (8) uses normal control diets with the ratio $\text{Ca:P} = 1.5$ to 1.0. Medes (9) considers a normal Ca:P ratio 1.4 or 1.5. McCollum (10) has normal diets with the Ca:P varying from 1.0 to 2.0, based upon analyses of the rat. Osborne and Mendel (11) give the ratio of 1.7 based upon the analyses of milk. In their later work (12) they have used 2.0, as a result of histological findings. Variations in the Ca:P ratio which these various authors consider normal imply that for the normal rat this factor is of minor importance, and only in absence of vitamin D, are small differences in the mineral constituents important. All of these ratios of Ca:P vary between 1.0 and 2.0. At the time when our experiments were performed the ratio of 2.0 was the best empirical value available.

That the curative agent in rickets was supplied completely either by the vitamin in cod liver oil or by the property conferred by irradiation, has been generally assumed. If either was supplied the other was unnecessary. Huston and Lightbody (13) have, however, shown that the growth of rats fed upon diets which contained adequate amounts of cod liver oil was improved either by hydroquinone or by irradiation. Our work confirms theirs and extends it. Both studies show that light and cod liver oil added to the diet result in double the rate of growth produced by the addition of either alone. Accordingly, the additional factor supplied by irradiation is a limiting factor for growth.

The metabolism studies show that retention of calcium and phosphorus are proportional to food intakes. The comparison of our data for retention with that of other investigators is difficult, because data for the age, gain, or food intakes or vitamin content are lacking (Telfer (14), McClendon (15), Medes (9), Hess (16)), or because the salt content of the diet was purposely made abnormal (Haag and Palmer (17)). Boas (8) has reported two sets of metabolism experiments in which all the data are included. The calcium and phosphorus retention in her animals supplied with cod liver oil closely approximates ours. The average ratios of retention of Ca:P were 1.72, 1.73, and 1.35, when the ratio of Ca:P of the diet was 1.5, and 1.56 when the ratio of the diet was 1.0. The diminution of the ratios of Ca:P in retention in our group that received both irradiated food and cod liver oil (Diet E) probably indicates that with good growth more phosphorus is utilized for growth of the tissues. In confirmation of our previous work and in agreement with Orr, Holt, Wilkins, and Boone (18), the ratio of Ca:P in retention is proportional to the ratio of the same elements in the diet.

SUMMARY AND CONCLUSIONS.

The diet has been progressively improved from a rickets-producing diet toward normal by alteration in the salt composition and by the addition of vitamins. A corresponding improvement occurs in the growth of animals, in the character of the bones, and in the retention of calcium and phosphorus. This improvement is brought about primarily by the increase in food consump-

tion. The best results ensue when the diet is supplemented with cod liver oil and by irradiation.

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RICKETS IN RATS.

IX. pH OF THE FECES.*

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Zucker and Matzner (1) have shown that the feces of ricketic rats are alkaline, pH 7.4, and that during the cure by cod liver oil the reaction becomes acid, pH 6.2. This has been corroborated by Jephcott and Bacharach (2) and also recently by Redman, Willimott, and Wokes (3). The former have suggested that this alteration in pH be used as a test for the cure of rickets. Variable findings have been reported by Redman (4) for children. The question then arises: Would other diets or other curative agents give different results, or is this a general method for testing the cure of rickets in rats?

Procedure.—Rickets was produced by Zucker's diet, or by the diet of Steenbock and Black, Ration 2965, plus 10 per cent lard. The procedure has been previously described (5). The ricketogenic diet was fed to rats at the age of 28 days. They continued on this diet for 21 days. Animals fed on Zucker's diet were then given cod liver oil. Those fed on Steenbock's diet were divided into groups of four or five animals. Cure was effected by the addition of cod liver oil, irradiation of the corn, irradiation and cod liver oil, or by the addition of phosphate sufficient to make the phosphorus equal in weight to, or double the weight of the calcium. The resulting diets, per 100 gm., were equivalent to 530 cc. of 0.1 N alkali, to neutrality, or to 315 cc. of 0.1 N acid.

These animals were studied for their responses to galvanic currents (6) and when tested, feces were produced and collected

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in clean test-tubes. Feces from the cages were never used. Thus the material was known to be fresh and uncontaminated. The pH was determined within an hour by the electrometric method. The chain consisted of a saturated calomel electrode, saturated KCl, agar bridge, test solution, and a gold-plated electrode with quinhydrone. The system checked daily against known solutions within 0.02 pH. Duplicates in close agreement were always obtained. As a further check the solutions were compared by the colorimetric method. This check was made independently by the second investigator.

Results.

The cure of rickets in rats by cod liver oil, irradiated food, and phosphates has been demonstrated by blood serum determinations for calcium and phosphorus, histological preparations of the bones, the ash content of the fat-free bones, and the metabolism of calcium and phosphorus (7). In this study it was further verified by x-ray pictures. Except for those animals which received high phosphate additions to the food and showed only partial recovery, all showed healed rickets after 2 weeks upon the curative measure.

The pH of the feces is tabulated in Table I. The data represent the average values of pH for all observations on a given day. The acidity determinations were so close that the pH and not CH values were averaged. The feces of rats fed upon Zucker's diet, which had previously been alkaline, became acid on the 5th to 9th day after administration of cod liver oil. This confirms the work of previous investigators (1-3) with regard to the time required for, and the extent of, the change.

The feces of the animals which received Steenbook and Black's rickets-producing diet were alkaline. In this case also, in 5 to 9 days after the administration of cod liver oil, they became less alkaline and were practically neutral. The feces of the group which received irradiated food did not show the change, but remained alkaline. With a diet containing both irradiated corn and cod liver oil, the feces were practically neutral. The rats which were cured by the addition of acid phosphates produced acid feces beginning with the 2nd day. Those which ate the neutral diet had neutral or alkaline feces. Those which received alkaline diets produced alkaline feces.

TABLE I.
Fecal pH of Rats Cured of Rickets by Various Procedures.

Diet.	Ca:P in diet.	Potential acidity of diet.	Days on diet.	Average pH.
Zucker.	4.73	cc. 0.1N per 100 gm. 109 alkali.	14	8.1
			21	7.6
Zucker + cod liver oil.	4.73	109 alkali.	1	8.17
			2	7.76
			3	7.70
			5	6.53
			7	6.77
			8	6.41
Steenbock.	6.65	530 alkali.	14	7.41
			20	8.00
			22	7.43
Steenbock + cod liver oil.	6.65	530 alkali.	4	7.02
			6	6.93
			13	7.00
Steenbock irradiated.	6.65	530 alkali.	4	7.15
			6	7.45
			13	7.32
			17	7.68
Steenbock irradiated + cod liver oil.	6.65	530 alkali.	4	6.83
			5	7.18
			6	7.27
			9	7.28
			11	6.92
Steenbock neutral + phosphate.	0.90	35 alkali.	1	7.89
			2	7.08
			3	7.80
			4	7.48
Steenbock neutral + high phosphate.	0.50	15 alkali.	2	7.69
			3	7.60
			5	7.09
			7	7.08
			9	7.18

TABLE I—*Concluded.*

Diet.	Ca:P in diet.	Potential acidity of diet.	Days on diet.	Average pH.
Steenbock acid + phosphate.	0.90	cc. 0.1N per 100 gm. 350 acid.	1	7.55
			2	6.85
			3	6.88
			4	6.55
Steenbock acid + high phosphate.	0.50	350 acid.	1	7.76
			2	7.73
			3	7.31
			5	6.90
			7	6.85
Steenbock alkaline + phosphate.	0.90	860 alkali.	1	7.60
			2	7.21
			3	7.43
			5	7.04
			7	7.06
			9	7.48
			13	7.28

DISCUSSION.

The confirmation in several laboratories of Zucker's observation is interesting. However, as has recently been suggested by Coward (8), it seems hazardous to use the acidity of the feces as a test of antiricketic potency, even for cod liver oil preparations. The slight alteration in pH with Steenbock's diet as compared with the greater shift with Zucker's diet may be associated with their different potential alkalinities. The former, per 100 gm., is equivalent to 530 cc. of 0.1 N and the latter to 110 cc. of 0.1 N alkali.

Because the cure can be accomplished also in other ways than by ingestion of cod liver oil, the test is further limited. The published statements concerning the effect of irradiation are not necessarily in conflict with our own observations. Zucker (1) and also Jephcott and Bacharach (2) irradiated the rats as a preventive measure and found a constant acid pH. The ricketic rats of Jephcott and Bacharach that were irradiated showed "the

change in faecal pH illustrated" in their chart. The graph shows only a temporary drop below pH 7.0 for each of the two pairs of rats, and thereafter the feces are alkaline. Further, their findings cannot be compared directly to ours because they used Zucker's diet, which on irradiation, may result in a greater change in fecal pH than would a more alkaline diet.

The cure of rickets is not necessarily associated with an alteration of the pH of the feces from alkaline to acid. The laws which govern the reaction of the feces are not well understood. The calcium, phosphorus, and fat content must be important (9). Certainly, the effect of variations in the acid-base value of the diet on pH of the feces must be taken into account.

SUMMARY.

Alteration in the feces from alkaline to acid has been observed when rats fed on Zucker's diet are cured of rickets by cod liver oil. Although the change is of great interest in interpreting the mechanism of the action of cod liver oil, the cure due to other agents may not render the feces acid. This change does not occur when rats made ricketic on Steenbock's diet are cured by irradiation of the food or by the addition of alkaline phosphates.

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A COMPARISON OF THE BERGEIM AND STANDARD METHODS OF DETERMINING COEFFICIENTS OF - UTILIZATION WITH SUGGESTED MODIFICATIONS.

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The customary proximate food analysis often fails to serve as an index of the feeding value of a ration. More satisfactory methods, such as the determination of the coefficient of utilization of any particular constituent of the feed, have long been recognized. The progress in the improvement of the method since its introduction by the German workers, and the frequent discussions of its application are so well known that a review of the literature at this time is not necessary. Armsby (1), who has made extensive application of these methods in this country, has outlined the most satisfactory steps of procedure. These methods are not used so extensively, however, as the information obtained would warrant, because of the tedious work involved as well as the many opportunities for error; namely, determining the accurate amount of food eaten, obtaining an equilibrium in the animal's body, collecting the total amount of feces and urine, and the preserving of the samples until the time of analysis. Bergeim (2) has recently suggested a method which eliminates many of the long, inaccurate steps of the old methods, and one, which if found to correlate well with the previous methods, might find extensive application. This method briefly consists in adding an insoluble iron compound to the ration and calculating the utilization coefficient on the basis of the ratio between the percentage of iron and the desired constituent in the feed, compared to that of the same two substances in the feces. For certain studies that we were attempting to make, this method would have proved far

superior if we had been sure that our results were comparable with the standard method, but an investigation of the literature failed to provide such information. In order to check these points we have attempted to make these determinations on a large number of animals, running the two methods in parallel, noting particularly whether the coefficient of utilization calculated by the two methods were comparable; second, whether the iron were all eliminated in the feces; third, whether a period of time were necessary after starting the feeding tests before the animal's body reached an equilibrium with the iron; and fourth, whether the utilization of the animal might be affected by the presence of the iron, an element, the salts of which are often administered for therapeutic purposes. Bergeim (2) bases his method on the commonly accepted view that ferric oxide is eliminated quantitatively in the feces and that it has no effect upon assimilation. On the other hand, Wada (3) states that possibly ferric oxide has the power to increase assimilation.

EXPERIMENTAL.

In order that the two methods could be accurately compared, it became necessary to choose test animals that could be kept under constant observation, and that their food and water should not be contaminated by contact with iron or its compounds. The rat was chosen as the most desirable animal. Metabolism cages containing no iron in their construction were next provided by cylindrical cages built of glass tubing set in concrete rings. The cage bottoms were similarly built of glass rods set in cement, the rims being enameled. Under this floor was placed an aluminum screen to catch the feces, while the urine was gathered in a porcelain dish below the aluminum screen. The greatest obstacle, that of a feeding jar from which feed could not be wasted, was overcome by securing a jar 6 inches long and $1\frac{1}{2}$ inches in diameter at the top. About the top was a 3 inch extension flange which offered a seat for the rats while eating and which caught any feed removed. The entire feeder was suspended by copper wire. In order to eat, the animal had to sit on this swinging seat and crawl head first into the jar. The position made it impossible to scratch out any food, and it was the only feeder ever used by the writer from which food was not wasted.

Distilled drinking water was provided through glass tubes from inverted bottles suspended on the outside of the cage. The animals used were healthy young males, females being omitted due to possibility of abnormal factors produced in pregnancy. In all cases the animals selected were kept on the ration being studied for several weeks prior to the time of the collecting of the first samples, so that a condition of equilibrium would be obtained in the animal's body and also to overcome any abnormalities that might be produced by a change in ration or quarters.

As previously stated, the work was so planned that the coefficient of utilization of the proteins, fats, fibers, and minerals could be determined by the Armsby method, the total feed and feces being analyzed, and at the same time the comparison of the coefficient of utilization based on the iron content of the feed and feces checked by the Bergeim method. In a parallel series the same determinations were being made, iron in the form of 0.5 per cent ferric oxide being added to the ration, the object being in this case to determine whether the presence of the iron had any effect on the utilization of the feed.

In the course of the study, hundreds of determinations have been made, a wide variety of rations as well as various iron salts being used. In this report, however, accounts will be made of rations similar in composition to that used for feeding purposes generally (Ration I); and of those similar in nature but having a protein supplement of more definite composition (Ration II).

The constituents of the rations and their analyses are as follows:

<i>Ration I.</i>		<i>Ration II.</i>	
	<i>parts</i>		<i>parts</i>
Yellow corn.....	60	Yellow corn.....	50
Whole wheat.....	28	Whole wheat.....	45
Tankage.....	5	Casein.....	10
Alfalfa leaves.....	5	Cod liver oil.....	2.5
NaCl.....	1	NaCl.....	1
CaCO ₃	1	CaCO ₃	1
<i>Analysis of Ration I.</i>		<i>Analysis of Ration II.</i>	
	<i>per cent</i>		<i>per cent</i>
Protein.....	14.93	Protein.....	16.8
Fat.....	2.99	Fat.....	4.44
Ash.....	7.19	Ash.....	2.85
Fiber.....	3.51	Fiber.....	2.6
Water.....	9.96	Water.....	8.6
Carbohydrates.....	61.42	Carbohydrates.....	64.71

It was soon found that the animals did not consume the same amounts of food daily and that the weight of feces did not parallel the food intake. This was due, no doubt, to changes in room temperatures and other factors. This has been noted of the food intake of an entire series of animals under experimental observation. For this reason, it was found advisable to take the food intake of a 4 day period and mix the feces and urine of the same period, although the samples were collected and preserved daily to prevent decomposition. The methods of analysis for protein, fat, moisture, ash, and fiber were those customarily used in feed control work and as adopted by the Association of Official Agricultural Chemists (4). In the case of iron determinations, however, many methods have been tried and various modifications introduced in an attempt to find some method that would be simple and at the same time uniformly accurate. The method recommended by Bergeim (2) was first used. Briefly stated it consists in taking a given weight of the sample and ashing at black heat, dampening with nitric acid and reashing, dissolving the residue in 5 cc. of 1:1 HCl and diluting to 10 cc., oxidizing with permanganate, adding 1 cc. of 2 per cent sodium thiocyanate, and comparing the color produced with a standard iron sample similarly and simultaneously prepared. This method has its limitations, and while its use has been checked on hundreds of determinations, it has been found usually to be low, especially in the analysis of the feces from the iron-consuming rats. It was first thought that the animals might be storing the iron or it might be excreted in the urine, but an analysis of the organs of the rats, and in fact of the whole animal, did not account for this loss. It was found that when the crucible in which feed was ashed was heated too rapidly the crucible often fused with some of the iron, while ashing at lower temperatures often left some organic matter. The method of Elvehjem and Hart (5) was next used on the assumption that perhaps some substance might be present that caused a fading of the colors produced. This method, while undoubtedly more accurate, especially for examining animal tissue, proved too tedious for the large number of analyses necessary for the proposed work, and it has been quite definitely proved since that time that fading was not the difficulty in the Bergeim method as applied to feed and feces. A modification of the

Kennedy (6) method has proved to be the most rapid and accurate and has been adopted for this study. As applied, it requires the following procedure: 1 gm. of the feed or 0.25 to 0.5 gm. of the dried feces are digested in a 250 cc. Kjeldahl flask with 5 cc. of iron-free sulfuric acid and 2 cc. of perchloric acid. A rack has been prepared so a large number of samples can be handled at the same time. 15 minutes is usually sufficient for complete digestion. The solution is then diluted to 50 cc. and a dilute permanganate solution is added slowly until fully oxidized. It has been found that in many cases considerable oxidation seems necessary even though the perchloric acid has been fully removed. The solution is then transferred with many washings to 100 cc. flasks and diluted to the mark. Aliquot portions are removed to large test-tubes and treated with 5 cc. of 20 per cent sodium cyanate and the color compared at once in a colorimeter with equal aliquots of standards containing varying amounts of iron which have been similarly and simultaneously prepared. These standards are prepared by introducing varying amounts of an iron standard containing 0.1 mg. of iron per cc. in the form of ferric ammonium sulfate.

Complete analysis of each lot of feed, and many times of the feed left in the feeder, together with that of the feces and urine, has been made after each 4 day period from several lots of animals over intervening periods during a 5 months period.

In order to avoid the presentation of an unnecessarily large amount of data, and also to overcome the variations creeping into short time observations, the average analyses of 16 day periods have been calculated and recorded in Table I. It is to be noted, however, that at least four animals were used in each test and a complete analysis carried out at the end of each 4 day period. The even numbers in every case represent the coefficients of utilization of the various constituents, calculated from the amount of these materials found in the feed and feces of a corresponding period. The second column of Table I gives the percentage of iron recovered, calculated from the theoretical amount of iron consumed in the amount of food eaten and the amount actually found in the feces for a corresponding period of time. The coefficients of utilization were calculated by the two methods in the usual way, the results being given in the other columns of the table.

The even numbered samples in every case represent the findings from animals receiving iron only as a normal constituent of the ingested food, the coefficient of utilization being calculated by the Bergeim method on the basis of the normally occurring iron of the feed. The odd numbered samples represent the analysis of a second series of rats fed exactly the same rations, to which have been added varying amounts of ferric oxide, and observed under exactly the same conditions and at the same time as those maintained for the previous lots. It should be stated that these

TABLE I.
Coefficients of Utilization.

Sample No.	Iron recovered.	Standard method, per cent.					Bergeim method, per cent.				
		Protein.	Ether extract.	Ash.	Fiber.	N-free extract.	Protein.	Ether extract.	Ash.	Fiber.	N-free extract.
	<i>per cent</i>										
1*	75.18	65.8	77.25	39.9	31	88.25	57.29	67.63	35.92	28.12	83.04
2	97.18	66.7	74.15	39.6	30.5	89	63.7	79.3	32	34.25	89.19
3*	65.05	68.5	76	41.25	33	90.5	64.77	60.44	29.55	21.67	89.21
4	97.05	64.5	71.15	33.1	37.75	90.5	64.15	73.95	33.1	32.50	90.72
5*	92.4	60.5	74	35.95	33.15	87.35	58.90	77.29	30.56	30.51	86.46
6	97.72	59.85	76.37	35.4	28.9	82.75	62.12	82.88	34.3	40.7	88.08
7*	89.37	52.1	75.55	34.7	25.3	88.1	45.75	71.49	25.5	24.68	85.66
8	97.03	56.1	76.35	32.32	21.32	86.45	55.43	75.33	22.85	22.14	86.10
9*	91.92	47.86	88.1	20.35	19.80	89.71	49.11	91.90	13.84	15.59	83.63
10	98.17	52.45	86.5	19.95	21.2	89.27	50.72	86.06	21.24	17.61	85.74

* Ferric oxide added to the ration.

animals consumed above 5 times as much iron as those eating the basal ration.

Table I presents interesting data requiring some comment. First, it is to be noted that the coefficients of utilization by the two methods correspond surprisingly well for the rations containing no added iron. It has been commonly conceded that iron is excreted largely through the feces, and even if assimilated, it was again excreted into the intestine. If this is true, the Bergeim method might be applied to the rations to which no iron was added and thus be more successful than when first considered, as it would eliminate the criticism of possibly altering the assimila-

tion of food due to possible therapeutic action and also to a very valid criticism that has been observed and will be mentioned later. This would necessitate, however, the use of a ration containing sufficient iron always to meet the needs of the animal and maintaining the animal on this particular ration until the animal's body had established an equilibrium with respect to iron. The analysis of the urine has been made often through the study; but the results are omitted, as it has been found that the amount of iron is so insignificant as not materially to affect our studies. The coefficient of utilization of the ash is obviously merely the measure of the minerals escaping in the feces as compared to that in the urine and is no more variable than might be postulated under the circumstances.

Next, on examination of the data for the animals listed under even numbered samples and consuming iron-supplemented rations, it will be noted that the iron content of the feces of the first two of these does not account for a large percentage of the ingested iron. This presented the greatest obstacle of the study. An analysis of animals failed to reveal any iron storage to explain the loss. The urine was only slightly higher in iron content. Each of the methods tried, notwithstanding all precautions, failed. Finally the analysis of the feed left in the feeders explained the greatest loss, and this incidentally offers the greatest criticism to the method. Despite the fact that the entire ration was finely ground and thoroughly mixed, it was found that the iron oxide had a tendency to separate out, and to what extent the animals were able to effect the separation cannot be stated. To overcome this difficulty the feed was dampened and baked into biscuits, but such a method is not applicable in many cases, and undoubtedly the digestibility of the feed must be so altered. The later determinations were made more nearly successful, so far as the recovery of iron was concerned, by mixing cod liver oil with the ration, and this had a tendency to bind the oxide to the feed. As a further precaution only as much food was provided as eaten, relatively small amounts being left at the end of the feeding periods. Despite all precautions, there was noted invariably some loss of iron in the feces of the animals consuming the iron-supplemented rations. This obstacle would become greater in preparing feed for large animals.

From the above data it would seem that a fairly accurate and at the same time a much more rapid method of determining the coefficients of utilization can be carried out as follows: A uniform mixture of the food to be studied, without the addition of extra iron, is prepared and fed for a period of 2 weeks prior to the time of analysis. Samples of the feces are gathered daily over a considerable period of time. The same are rapidly dried and preserved. 1 gm. samples of feed and 0.5 gm. samples of feces are analyzed for iron by the modified Kennedy method as previously indicated. The other constituents of feed and feces are analyzed by the customary methods and the coefficients of utilization calculated by the original method suggested by Bergeim.

DISCUSSION.

1. Standard utilization studies are accurate only within limits and are so time-consuming as to be of value only in limited studies.

2. The Bergeim method may be applied in utilization studies, as results so obtained are comparable to the older methods, providing indicated precautions are observed.

3. Some iron is usually not recovered when added to the ration.

4. It is exceedingly doubtful whether the iron oxide would be eliminated in a like proportion in all parts of the feces, thereby making possible the sampling of any portion at random during the day.

5. The indications are that most rations contain sufficient iron to apply the Bergeim method without the addition of the ferric oxide. Such procedure should provide more uniform intake and a more evenly distributed iron excretion in the feces.

6. The method suggested, the normal iron content of rations being used, retains the advantage of the original Bergeim method and eliminates the unfavorable steps of that method.

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A COMPARISON OF RAW, PASTEURIZED, EVAPORATED, AND DRIED MILKS AS SOURCES OF CALCIUM AND PHOSPHORUS FOR THE HUMAN SUBJECT.*

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It has been generally recognized that milk serves as an excellent source of calcium and phosphorus in the diet. However, an increasing tendency toward the use of such forms as evaporated, dried, and especially pasteurized instead of raw milk makes it seem of value to determine whether or not the same reliance can be placed upon all forms of milk as sources of the minerals, calcium and phosphorus. Experiments here described were planned to add to the present rather limited knowledge concerning the availability of calcium and phosphorus from various forms of milk, fresh raw milk being used as a standard and both children and adults as subjects.

LITERATURE.

A search of the literature fails to reveal many metabolism experiments with the human subject for the study of the problem at hand. Furthermore, almost none of the experiments had a definite standard for comparison.

Willard and Blunt (1) have reviewed the more important studies made with both human and animal subjects; namely, the work of Washburn and Jones (2), Magee and Harvey (3), Daniels and Loughlin (4), and Daniels and Stearns (5). These investigations point to the superiority of raw, evaporated, condensed, and quickly boiled milks over pasteurized milk. Willard and Blunt (1) made an extensive balance study, using pasteurized and evaporated milks. The children used as subjects all showed better retention of calcium and phosphorus when evaporated rather than pasteurized milk was used. Increased retention on evaporated milk was shown by two of their four adult subjects.

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Ladd, Evarts, and Franks (6) made a study of the relative efficiency of certified and pasteurized milk for infant feeding by following the gains in weight made by a large number of subjects. They found that the certified milk allowed for better gains than the pasteurized, even when orange juice and cod liver oil were added. They raise the question whether the superiority of the certified milk is due merely to the fact that it is raw or to the better balanced feeding of the cows.

Differences in availability of the minerals in the food seems even more important in the light of the report of Hart, Tourtellotte, and Heyl (7) whose adult subject showed no increase in calcium retention as the result of irradiation or as the result of the administration of cod liver oil.

EXPERIMENTAL.

Calcium and phosphorus balance experiments were carried out in three series, both children (Series 1, conducted in Topeka) and adults (Series 2 and 3, conducted in Manhattan) being used as subjects. The same general plan was used throughout, changes being made only where experience indicated possible improvements. In all cases every effort was made to have milk the only variable in the experiment. Simple diets were planned, adequate in protein (8) and calories. Milk was made to furnish as much of the total calcium as possible. Milk also furnished much of the phosphorus, but its composition is such that it could not provide equally large proportions of both elements. In order that differences might show, it seemed essential to keep the total intake of calcium and phosphorus near the minimum required for maintenance in the adults (8) and below the amount required for optimum storage in the children.

The children were given about 625 gm. of fresh milk daily and equivalent amounts of dried milk, this being below the figure for optimum storage suggested by Sherman and Hawley (9). Fresh milk furnished not less than 75 per cent and dried milk not less than 73 per cent of the total calcium. Almost half the phosphorus was supplied by the milk.

The adults in Series 2 received 222 gm. of fresh milk daily, with calculated equivalent amounts of the other forms during the various periods. Analyses later showed that the subjects received about 0.56 gm. of calcium per day. The milk furnished 47 to 52 per cent of the total calcium but only about one-fourth of the total phosphorus. In Series 3 the fresh milk was increased to 260 gm. per day, but the diet was so changed as to bring the total calcium down

to 0.50 gm. per day, which is nearer the minimum of 0.45 gm. set by Sherman (8). The milk furnished 68 per cent of the total calcium and 38 per cent of the total phosphorus.

As much as possible foods were chosen which could be purchased in quantity to last through an experiment so that the composition would not vary.

A standard brand of dried milk made by the spray process and also a popular brand of evaporated (*i.e.* unsweetened condensed) milk were chosen as examples of these products now in common use. These same commercial brands were used in all experiments. In Topeka the fresh milk was obtained from a commercial dairy marketing a product of very high grade. The fresh milk for Series 2 and 3 was obtained from the College Dairy. For Series 2 it happened that milk was available from two cows which had been kept in the dairy barn away from the sunlight for some months before coming fresh. The cows had been in the barn 5 months at the time the milk (called Fresh Milk B) was used for the experiments. The cows producing this milk were of the college herd and were receiving the same rations as the animals in the remainder of the herd from which the fresh milk for the experiments was obtained. For Series 3 milk pasteurized by the "hold" process was obtained from the College Dairy. It was pasteurized in the morning and used at noon. It was of exactly the same origin as the fresh milk used in the same series.

All experiments were divided into 3 day periods. Foods were weighed and records kept of daily consumption of subjects. Food samples of approximately the same size were taken daily, weighed, and dried or, in the case of liquids, preserved with formaldehyde for analysis. All samples of urine and feces were collected, carmine being used to mark the feces of the various periods.

The McCrudden (10) method of analysis was used for calcium determinations of both food and feces for all series. Dry samples were ashed in an electric muffle furnace, and liquid samples with nitric acid.

For samples of Series 1 and 2 the phosphorus determinations were made by the gravimetric method of double precipitation, first as ammonium phosphomolybdate and then as magnesium ammonium phosphate. A volumetric method recommended by the Association of Agricultural Chemists (11) was used for deter-

mination of phosphorus in samples for Series 3. Magnesium nitrate was used for ashing. The methods used were tested for accuracy on samples of known composition. Analyses were made with the cooperation of the chemists of the Agricultural Experiment Station and the methods were those used by them in routine analyses. All analyses were made in duplicate.

Since the content of calcium and phosphorus varies within wide limits for most foods, analyses were made of all foods used, no figures being accepted from previously compiled tables (Table I).

TABLE I.
Food Analyses (Percentage of Edible Portion).

	Series 1.		Series 2.				Series 3.	
	Ca	P	First six periods.		Last two periods.		Ca	P
			Ca	P	Ca	P		
Fresh milk.....	0.124	0.024	0.127	0.023	0.127	0.023	0.134	0.105
Fresh Milk B.....					0.125	0.025		
Pasteurized milk.....							0.134	0.104
Dried milk.....	0.915	0.181	0.976	0.176			0.934	0.768
Evaporated milk.....			0.256	0.046			0.283	0.210
Apple.....	0.033	0.004						
Bread.....	0.012	0.020	0.052	0.019	0.051	0.026	0.050	0.092
Butter.....	0.017	0.004	0.028	0.004	0.030	0.004		
Cocoa.....	0.032	0.184						
Rolled oats.....	0.136	0.106						
Orange juice.....	0.012	0.006	0.014	0.052	0.018	0.005	0.008	0.014
Potato.....	0.022	0.016	0.032	0.018	0.026	0.014	0.006	0.042
Prunes.....	0.028	0.031	0.078	0.016	0.082	0.025	0.055	0.095
Lean ground beef.....			0.030	0.051	0.018	0.050	0.016	0.223

Series 1 (Started in October, 1924).—Three girls and two boys, 7 to 12 years of age, served as subjects for this series. The feeding experiments were conducted in the Topeka Home of the Kansas Children's Home Society where necessary facilities for the work were kindly provided. The children were examined by a physician, a child specialist, and were pronounced suitable for the investigation. The experiment was divided into the following periods: Period I, fresh milk; Period II, fresh milk; Period III, dried milk; Period IV, dried milk; Period V, fresh milk.

The regular hours of the institution were maintained for the

children used as subjects. Time spent in the open air, including walks to and from the public school a few blocks distant, remained practically uniform throughout the experiment. During the entire time only 2 days were cloudy, sunny fall weather typical of the region prevailing during the remainder of the time. The

TABLE II.

Series 1.

Eunice, female, age 12 yrs., weight 38.2 to 41.1 kilos.

Period No.....	I	II	III	IV	V
Average food intake for each period, in gm. per day.					
Fresh milk.....	662	625			625
Dried "			82	82	
Apple, E. P.*.....	208	200	200	217	200
Bread.....	318	299	384	325	299
Butter.....	90	110	110	150	93
Cocoa.....	5	7	7	7	4
Oats.....	40	45	45	45	45
Orange juice.....	57	65	65	65	65
Potato, E. P.....	198	250	250	250	250
Prunes, "	25	50	50	50	50
Sugar.....	27	35	27	20	22
Calories.....	2759	2919	3093	3232	2724
Protein.....	66	66	74	69	65
Calcium.....	1.051	1.030	1.018	1.023	1.026
Phosphorus.....	0.657	0.684	0.711	0.692	0.672

Ca and P balances in gm. per day.

Ca output.....	0.433	0.699	0.785	0.626	0.572
" balance.....	+0.618	+0.331	+0.233	+0.397	+0.454
P output.....	0.254	0.272	0.374	0.352	0.354
" balance.....	+0.403	+0.412	+0.337	+0.340	+0.318

* E. P., edible portion.

nutritive condition of the children remained good throughout and each subject showed a satisfactory gain in weight.

The diet contained, daily, 625 gm. of fresh milk or its calculated equivalent in dried milk. Other foods were apples, cocoa, orange juice, prunes, potatoes, rolled oats, sugar, bread, and butter. The last two items were given in amounts desired but the other foods were kept nearly constant (Table II).

Series 2 (Started in February, 1926).—Four healthy young women who were instructors and graduate students served as subjects for the second series of experiments. The periods of the

TABLE III.

Series 2.

V. C., female, weight 63 kilos.

Period No.....	I	II	III	IV	V	VI	VII	VIII
Average food intake for each period, in gm. per day.								
Dried milk..	30	30						
Fresh " ..			222	222				
Evaporated milk.....					100	100		
Fresh Milk B.....							222	222
Bread, white.....	250	250	250	250	250	250	230	230
Butter.....	100	100	100	100	100	100	70	70
Orange juice.....	200	200	200	200	200	200	200	200
Potato, E. P.*....	120	120	120	120	120	120	120	120
Prunes, E. P.	50	50	50	50	50	50	50	50
Beef, lean..	80	80	80	80	80	80	80	80
Sugar.....	90	90	90	90	90	90	70	70
Calories....	2391	2391	2392	2392	2406	2406	2029	2029
Protein.....	52	52	52	52	55	55	50	50
Calcium....	0.580	0.580	0.568	0.568	0.543	0.543	0.538	0.538
Phosphorus.	0.373	0.373	0.371	0.371	0.360	0.360	0.393	0.393

Ca and P balances in gm. per day.

Ca output..	0.686	0.680	0.580	0.581	0.536	0.545	0.650	0.655
" balance..	-0.106	-0.100	-0.012	-0.013	+0.007	-0.002	-0.112	-0.117
P output...	0.388	0.383	0.371	0.369	0.361	0.369	0.534	0.526
" balance..	-0.015	-0.010	0.000	+0.002	-0.001	-0.009	-0.141	-0.133

* E. P., edible portion.

experiment were as follows: Period I, dried milk; Period II, dried milk; Period III, fresh milk; Period IV, fresh milk; Period V, evaporated milk; Period VI, evaporated milk; Period VII, fresh milk B; Period VIII, fresh milk B.

A diet was planned to follow closely that of the children, the calcium being reduced to the point where differences might be expected to show. The diet contained white bread, butter, orange juice, potatoes, prunes, lean ground beef, and sugar. Body weights of subjects were kept constant by adjusting the amounts of butter and sugar in the diets. Subjects remained indoors as

TABLE IV.
Series 3.

I. B., female, weight 58.2 kilos.

Period No.....	I	II	III	IV	V	VI
Average food intake for each period, in gm. per day.						
Pasteurized milk.....	260	260				
Raw milk.....			260	260		
Dried milk.....					34	34
Bread.....	200	200	200	200	200	200
Butter fat.....	90	90	90	80	80	80
Orange juice.....	200	200	200	200	200	200
Potato, E. P.*.....	120	120	120	120	120	120
Prunes, ".....	35	35	35	35	35	35
Beef.....	80	80	80	80	80	80
Sugar.....	80	80	80	70	70	70
Calories.....	2244	2244	2244	2034	2027	2027
Protein.....	48	48	48	48	48	48
Calcium.....	0.503	0.503	0.503	0.503	0.472	0.472
Phosphorus.....	0.711	0.711	0.711	0.711	0.702	0.702

Ca and P balances in gm. per day.

Ca output.....	0.632	0.619	0.525	0.453	0.530	0.532
" balance.....	-0.129	-0.116	-0.022	+0.050	-0.058	-0.060
P output.....	0.813	1.057	0.692	0.800	0.693	0.766
" balance.....	-0.102	-0.346	+0.019	-0.089	+0.009	-0.064

* E. P., edible portion.

much as possible, so that the factor of exposure to sunlight was almost eliminated (Table III).

Series 3 (Started in February, 1928).—Four healthy young women who were instructors and graduate students served as subjects for the third series of experiments, which was conducted in the late winter as was the second series. The general plan of the experiments was much the same. The periods were: Period I,

pasteurized milk; Period II, pasteurized milk; Period III, fresh milk; Period IV, fresh milk; Period V, evaporated milk for two subjects and dried milk for two subjects; Period VI, evaporated milk for two subjects and dried milk for two subjects.

Profiting by the previous experiments, we made a few changes. In the first place, the diet was so planned that a larger per cent of the total calcium would be furnished by the milk. The total intake of calcium was somewhat lower than in Series 2, bringing

TABLE V.

Series 1. Calcium and Phosphorus Balances in Gm. per Day (Average of Periods Used).

Subject.	Kind of milk.	Calcium.				Phosphorus.			
		In-take.	Out-put.	Balance.	Balance per kg.	In-take.	Out-put.	Balance.	Balance per kg.
Eunice, 12 yrs., 40 kilos.	Fresh.	1.032	0.568	0.464	0.012	0.671	0.293	0.378	0.009
	Dried.	1.025	0.706	0.319	0.008	0.702	0.363	0.339	0.008
Jack, 9 yrs. 8 mos., 32 kilos.	Fresh.	1.066	0.761	0.305	0.010	0.654	0.391	0.263	0.008
	Dried.	0.939	0.732	0.207	0.007	0.628	0.473	0.155	0.005
Lottie, 8 yrs. 3 mos., 26 kilos.	Fresh.	1.043	0.493	0.550	0.021	0.682	0.372	0.310	0.012
	Dried.	0.991	0.596	0.395	0.015	0.666	0.425	0.241	0.009
Nellie, 10 yrs. 6 mos., 28.3 kilos.	Fresh.	1.037	0.325	0.712	0.025	0.652	0.262	0.390	0.014
	Dried.	0.978	0.603	0.375	0.013	0.639	0.357	0.282	0.010
Wayne, 7 yrs. 4 mos.	Fresh.	1.046	0.767	0.279	0.011	0.684	0.402	0.282	0.012
	Dried.	1.002	0.828	0.174	0.007	0.664	0.388	0.276	0.011

the figure nearer the minimum requirement of 0.45 gm. suggested by Sherman (8). Also, more rigid rules were set up for the consumption of food by the subjects. All subjects ate the same foods at the same time of day. All milk was consumed, without heating, as a beverage at the noon meal. The pasteurized and raw milks, which came from the College Dairy, were never older than of the milking of the previous night.

All subjects remained indoors as much as possible, although there was little sunny weather during the time of the experiment.

TABLE VII.
Series 3. Calcium and Phosphorus Balances in Gm. per Period (Average of Periods Used).

Subject.	Kind of milk.	Calcium.				Phosphorus.			
		Intake.	Output.	Balance.	Balance per kg.	Intake.	Output.	Balance.	Balance per kg.
E. L., weight 53.6 kilos.	Fresh.	1.474	1.234	+0.240	+0.004	2.064	2.050	+0.014	0.000
	Dried.	1.382	1.653	-0.271	-0.005	2.038	2.048	-0.010	0.000
	Pasteurized.	1.509	2.236	-0.727	-0.014	2.133	2.551	-0.418	-0.008
I. B., weight 58.2 kilos.	Fresh.	1.509	1.468	+0.041	+0.001	2.133	2.238	-0.105	-0.002
	Dried.	1.417	1.593	-0.176	-0.003	2.107	2.188	-0.081	-0.001
	Pasteurized.	1.509	1.876	-0.367	-0.006	2.133	2.804	-0.671	-0.012
A. L., weight 68 kilos.	Fresh.	1.509	1.881	-0.372	-0.005	2.133	2.032	+0.101	+0.001
	Evaporated.	1.525	1.556	-0.031	0.000	2.110	2.092	+0.018	0.000
	Pasteurized.	1.509	1.883	-0.374	-0.006	2.110	2.092	+0.018	0.000
M. K., weight 56.3 kilos.	Fresh.	1.509	1.550	-0.041	-0.001	2.133	2.236	-0.103	-0.002
	Evaporated.	1.525	1.318	+0.207	+0.004	2.110	1.835	+0.275	+0.005
	Pasteurized.	1.509	1.803	-0.294	-0.005	2.133	2.348	-0.215	-0.004

One subject only had received cod liver oil and that had been some weeks before the experiment started.

The foods selected for the diet were white bread, potatoes, ground lean round of beef, prunes, orange juice, sugar, butter fat, and milk. Quantities of butter fat and sugar were adjusted so that the subjects remained constant in weight (Table IV).

TABLE VIII.

Comparisons of Balances of Calcium per Kilo per Period of Subjects on Various Kinds of Milk.

The balance of each subject on raw milk was taken as his or her standard or zero for the comparisons tabulated below.

	Pasteur- ized.	Dried.	Evap- orated.	Fresh milk B.
Series 1.				
Eunice.....		-0.012		
Jack.....		-0.009		
Lottie.....		-0.018		
Nellie.....		-0.036		
Wayne.....		-0.012		
Series 2.				
M. D.....		-0.004	-0.003	
R. T.....		-0.002	-0.002	
V. C.....		-0.004	+0.001	-0.004
M. K.....		-0.004	+0.001	-0.003
Series 3.				
I. B.....	-0.007	-0.004		
E. L.....	-0.018	-0.009		
M. K.....	-0.004		+0.005	
A. L.....	-0.001		+0.005	
Averages.				
Series 1 (children).....		-0.017		
Series 2 (adults).....		-0.004	-0.001	-0.004
Series 3 (adults).....	-0.008	-0.007	+0.005	
For all adults.....	-0.008	-0.005	+0.001	-0.004

DISCUSSION.

The experiment with children showed good storage of calcium from fresh milk not only at the beginning of the experiment but at the end also when the fresh milk period was repeated. During

the dried milk periods the diets contained almost as much calcium and a little more phosphorus than during the fresh milk periods. However, figures show the calcium retention of the various children to have averaged only 53 to 71 per cent as much as during their fresh milk periods, although the dried milk periods furnished an average of 94.5 per cent as much calcium as did the fresh milk periods. Likewise the phosphorus retention was somewhat lower. It is thus evident that the child retains more calcium when it is supplied in fresh milk than when furnished in equal amounts by dried milk, other factors remaining unchanged (Table V).

The adult subjects in Series 2 and in Series 3 were at all times receiving close to the minimum amounts of calcium and phosphorus, sometimes showing negative balances and sometimes positive. (Tables VI and VII). Calculations have been made to show the calcium and phosphorus balances of each subject in gm. per kilo of body weight per period, averaged for like periods. The 3 day period was used, rather than the day, as the figures are necessarily rather small. Comparisons were then made, subject by subject, in each case the balance of a subject on fresh milk being taken as zero and the balances on other milks being listed as higher and lower or plus and minus in comparison with this fresh milk figure (Table VIII).

As with the children, dried milk gave for all subjects a lower calcium balance than did the fresh milk. Pasteurized milk, used in Series 3, gave a lower calcium balance than did fresh milk. On the other hand evaporated milk, used in both Series 2 and 3, gave a higher calcium balance for four of the six subjects. In Series 3, the most carefully controlled experiments, the subjects showed distinctly better calcium balances on evaporated milk than on fresh milk. In Series 2, Fresh Milk B, secured from cows kept in the barn for 5 months, gave far lower calcium balances than did the herd fresh milk. Utilization of calcium from this milk was only about as good as the utilization of calcium from the dried milk.

Since the amount of milk used furnished smaller proportions of the phosphorus of the diet, the results on phosphorus are necessarily less convincing. They follow in general the results for calcium.

SUMMARY AND CONCLUSIONS.

Metabolism experiments have been conducted with children and adults as subjects in an effort to learn whether or not the calcium and phosphorus in various forms of milk are equally available for human nutrition.

Results of experiments with five children make it evident that the child retains more calcium when it is supplied in fresh milk than when it is furnished in equal amounts by dried milk, other factors remaining unchanged.

All adult subjects showed more favorable calcium balances when the fresh milk was the source of supply rather than dried milk. Pasteurized milk also gave less favorable calcium balances than did fresh milk. Further, the milk from cows kept in the barn (Fresh Milk B) gave less favorable calcium balances than did fresh milk.

On the other hand adult subjects using evaporated milk showed balances at least as good as when fresh milk was used.

In general phosphorus balances followed the trend of the calcium balance figures.

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A COMPARISON OF pH DETERMINATIONS AS OBTAINED BY MEANS OF HYDROGEN ELECTRODE AND COLORIMETRIC METHODS.

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The wide application of studies of the hydrogen ion concentration of blood and other body fluids suggests the importance of a simple method for these determinations. The hydrogen electrode method, which is generally taken as standard, requires skill and a considerable amount of time and of blood. To overcome these disadvantages colorimetric methods have been devised, based on the comparison of colors given by an indicator when added to an unknown solution and to standard buffer solutions. Errors are encountered because the color of the indicator is influenced by proteins and salts as well as by the pH of the solutions. Cullen (1922, *a*) diluted serum twenty times with 0.9 per cent sodium chloride solution and concluded that the differences between the colorimetric and hydrogen electrode readings were sufficiently constant to employ a correction in order to convert colorimetric readings at 20° to the electrometric values at 38°. Hastings and Sendroy (1924) reported that this correction became zero when they compared colorimetric readings at 38° with the electrometric readings at 38°. But Bennett (1925-26) and Austin, Stadie, and Robinson (1925) compared the Cullen and the Hastings procedures with the electrometric (and gasometric) method and found the corrections to vary considerably.

A dialysis method for the determination of pH was originated by Levy, Rowntree, and Marriott (1915) and improved by Dale and Evans (1920-21). In this method blood is dialyzed and the indicator is added to the dialysate. The protein is practically completely absent from the solution tested but salts are present and

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will affect the colorimetric readings. It was thought desirable to study the dialysis method with the hope that the differences between the colorimetric and electrometric readings would be constant. Determinations were run by each of these colorimetric methods (the dialysis, the Hastings, and the Cullen methods) on the same blood and the results were compared with the value obtained by means of the hydrogen electrode.

EXPERIMENTAL.

Serum was used for the electrometric, the Cullen, and the Hastings methods. The whole blood for the dialysis method was obtained by allowing about 15 cc. to run into a tube containing oxalate, in the middle of the blood collection. The portion collected in this manner was assumed to be similar to the whole sample. In order to test the validity of this assumption several determinations were made by dialyzing the serum as well as the whole blood. The results in all cases were similar.

The blood used in these experiments was obtained from dogs. The femoral artery was exposed under cocaine, a cannula placed in the artery, and blood collected under oil in hard glass centrifuge tubes. After a sample was taken, the animal was allowed to bleed enough to make the total hemorrhage $3\frac{1}{2}$ to 4 per cent of the body weight and then a second sample was taken either immediately or after a period of 5 to 15 minutes. The dogs were rebled on the following day if they survived. At no time during the collection or handling of the blood was it allowed to come in contact with the air. Centrifuging was done under solidified paraffin and the serum drawn into hard glass tonometers over clean mercury as suggested by Austin, Cullen, *et al.* (1922).

The electrometric determinations were made at 38° with standard apparatus. The serum was placed in a Clark-Cullen electrode vessel and allowed to come to equilibrium with a carbon dioxide-hydrogen mixture (Cullen, 1922, *b*). Fresh portions of serum were introduced, the same gas mixture being kept until constant E.M.F. readings were obtained.

The Cullen colorimetric readings were made as described by Cullen (1922, *a*) except that a definitely measured amount of 0.02 per cent phenol red (0.1 cc.) was used instead of the 5 drops suggested by him. The sodium chloride solution was brought to pH

7.4 by passing a current of CO₂-free air through it (Earle and Cullen, 1928). The pH of the salt solution does not remain at 7.4 when put in the tubes under oil due to the absorption of traces of CO₂ into the unbuffered solution. It is, nevertheless, probably superior to the neutralized solution originally suggested. The Hastings readings were made in the same manner as the Cullen except that they were read with standards and unknowns at 38° instead of at 20°. The comparison tubes were made up from phosphate buffers, 0.05 pH apart, checked electrometrically at 38°.

The dialysis method was carried out as suggested by Dale and Evans (1920-21) with some modifications. Salt solution which had been brought to pH 7.4 as in the Cullen method was run into a small test-tube under oil. A collodion bag containing a few drops of oil was suspended in about 1.5 cc. of salt solution. Approximately 1 cc. of blood was then introduced into the bag under oil and allowed to dialyze at the desired temperature. It was not found necessary to stopper the tubes in which the dialysis was carried on if the blood and dialysate were kept under oil and stirring was minimized. This fact was ascertained by running a series with and without stoppers. Similar results were obtained in both cases. After dialysis for 30 minutes the sack containing the blood was removed and 0.1 cc. of 0.02 per cent cresol red solution was added. This was then compared with freshly prepared buffer standards 0.05 pH apart, containing the same amount of indicator. The temperatures of the dialysate and the standard were kept the same by means of a water bath.¹ The length of time for dialysis was arrived at by dialyzing portions of blood for varying lengths of time at 20°. It was found that although the samples dialyzed 15, 30, 45, or 60 minutes usually gave the same pH readings, occasionally a sample dialyzed for 15 minutes would give a reading lower than those dialyzed longer.

The collodion bags used were made from a 12 to 15 per cent solution of parlodion in equal parts of alcohol and ether. The solution was poured into test-tubes of appropriate size, drained for 2 minutes, and 70 per cent alcohol added (Gates, 1922). After 2 or 3 minutes the alcohol was poured off and the sacks loosened with distilled water. The sacks were then placed in a large vol-

¹ In the early experiments, certain readings were made at the prevailing room temperature as shown in Table I.

TABLE I.

Comparison of Results of Electrometric and Colorimetric pH Determinations.

Dog No.	Date.	Blood No.	Hydrogen electrode pH_{38° .	Dialysis methods.			Dilution methods.		
				Dialysis at room temperature.		Dialysis at 38° , $\text{pH}_{38^\circ} - \text{pH}_{38^\circ}$.	Cullen.		Hastings.
				Temperature.	$\text{pH}_c - \text{pH}_{38^\circ}$.		Temperature.	$\text{pH}_c - \text{pH}_{38^\circ}$.	
	1927			$^\circ\text{C}.$			$^\circ\text{C}.$		
13	Apr. 5	1	7.38	23	0.17	0.33 0.03			
16	" 15	1	7.42	19	0.28				
		2	7.62	19	0.18				
17	" 21	1	7.45	23	0.10				
		2	7.55	23	0.10				
	" 22	1	7.41	23	0.24				
13	June 16	1	7.40				22	0.34	0.10
		2	7.40				22	0.34	0.12
	" 17	1	7.41				21	0.18	0.04
		3	7.36				21	0.39	0.11
	" 22	1	7.35				24	0.40	0.10
		2	7.46				24	0.34	0.09
		3	7.27				24	0.48	0.18
	" 23	2	7.40				20	0.25	0.10
		3	7.44				20	0.36	0.06
26	" 29	1	7.32				20	0.45	0.13
		2	7.22				20	0.50	0.18
	" 30	1	7.32	25	0.28		20	0.22	0.06
		2	7.33	25	0.17		20	0.22	0.05
		3	7.43	25	0.42		20	0.42	0.27
		4	7.08	25	0.37				
		5	7.10	25	0.45		20	0.17	0.05
27	July 14	1	7.35	28	0.17				
		2	7.36	28	0.19				
	" 16	1	7.33	28	0.11		20	0.27	0.12
28	" 19	1	7.25	26	0.23		20	0.50	0.18
		2	7.32	26	0.23		20	0.23	0.0
	" 22	1	7.30	27	0.15		20	0.25	0.05
		2	7.30	27	0.35		20	0.25	0.05
29	" 26	1	7.27	27	0.13		20	0.45	0.13
30	Aug. 17	1	7.33	20	0.12		20	0.42	0.15
		2	7.35	20	0.10		20	0.40	0.10
		3	7.30	20	0.13		20	0.42	0.15

TABLE I—*Concluded.*

Dog No.	Date.	Blood No.	Hydrogen electrode pH _{38°} .	Dialysis methods.			Dilution methods.		
				Dialysis at room temperature.		Dialysis at 38° pH _{38°} - pH _{38°} .	Cullen.		Hastings.
				Temperature.	pH _c - pH _{38°} .		Temperature.	pH _c - pH _{38°} .	
	1927			°C.			°C.		
30	Aug. 18	1	7.32	20	0.28				
		2	7.44	20	0.36				
		3	7.00	20	0.35				
13	Sept. 14	1	7.37	20	0.13	-0.02	20	0.35	0.03
		2	7.31	20	0.24	0.04	20	0.36	0.04
		1	7.31	20	0.34	0.34	20	0.21	0.04
31	" 15	2	7.53	20	0.32	0.32	20	0.32	0.05
		1	7.35	20	0.25	0.10	20	0.37	0.10
		2	7.42	20	0.38	0.13	20	0.36	0.08
32	" 22	1	7.38	20	0.27	0.04	20	0.27	0.02
		2	7.67	20	0.28	-0.02	20	0.33+	0.08
		1	7.25	20	0.30	0.15			
33	Oct. 18	2	7.44	20	0.26	0.11			
		1	7.33	20	0.32	0.10			
		2	7.32	20	0.33	0.11			
34	Nov. 10	1	7.37	20	0.28	0.08	20	0.21	0.00
		2	7.52	20	0.38	0.13	20	0.23	0.06
		1	7.39	20	0.21		20	0.28	0.04
35	" 18	2	7.59	20	0.16		20	0.34	0.08
		1	7.39	20	0.39	0.28	20	0.26	0.00
		2	7.42	20	0.41	0.28	20	0.31	0.05

pH_{38°} indicates the pH determined at 38° by means of the electrometric (hydrogen electrode) method. pH_c indicates the pH determined by means of one of the colorimetric methods at the temperature shown. The corrections for the colorimetric methods are given as the differences between the colorimetric pH and the electrometric pH values. Only the differences are given as the colorimetric readings may be readily calculated by adding the differences shown in the table. Blood 1 was taken immediately before a severe hemorrhage. The others were taken at various times after hemorrhage.

ume of 0.9 per cent salt solution, and allowed to stand for 2 or 3 days before being used. They were washed well with salt solution before being used and were never used more than once. As parlodion develops some acidity on standing in water, solutions were made up from several samples to see whether those which were most acid would lower the pH readings obtained by the dialysis method. The readings that were made from the sacks prepared from the acid parlodion compared favorably with those from the sacks made of parlodion which was neutral in reaction. In accordance with the results obtained by Levy, Rowntree, and Marriott (1915) variations in thickness of sacks made no difference in readings. A comparison of the readings obtained by the methods studied is shown in Table I.

All determinations were done in duplicate except in the case of the dialysis method where a series of five separate determinations was made for each reading. All colorimetric tubes were brought to the desired temperature by means of a water bath.¹

DISCUSSION.

Cullen (1922, *a*) found a correction for his method of 0.35 pH for dog blood, and 0.23 pH for human blood to make colorimetric readings at 20° correspond to electrometric readings at 38°. Hastings and Sendroy (1924) reported a very good agreement between the colorimetric and electrometric readings when both were made at 38°. Our data show variations in the Cullen correction from 0.13 pH to 0.41 pH and variations in the Hastings correction from 0.00 to 0.18 pH, so that even the variations in the Hastings correction are sufficiently great to make the Hastings method too rough for accurate work.²

The data presented show the correction of the dialysis method to be inconstant. As in the case of the dilution method the correction diminishes as the temperature is raised from 20° to 38°. The differences between the readings at 20° and those at 38° are not constant so that it is not possible to read at one temperature

² Incidentally, these results confirm the observations of Miss Bennett that the Cullen correction becomes lower than normal the day after hemorrhage. In the present study the samples after hemorrhage were taken too soon to permit any appreciable variation to take place, though there is usually a slight rise in the correction.

and correct to another. The dialysates which had been read at 20° were then placed in a water bath at 38° and, after being allowed to reach the temperature of the bath, were read again at the higher temperature. The results were so variable that no conclusions could be drawn from them.

Evans (1921) found that the difference between the results of the dialysis method and the electrometric or gasometric method was about 0.20. This correction factor was obtained from studies on 0.02 M NaHCO_3 at varying CO_2 tensions. The electrometric and colorimetric determinations were both made at 20°. The individual corrections varied from 0.12 pH to 0.27 pH and the average of five experiments was just below 0.20 pH. For use with blood the method was checked on two samples in which the corrections were 0.15 and 0.20. Evans' original idea that the electrometric values were too low was subsequently shown to be incorrect. Bayliss, Kerridge, and Verney (1926) compared the dialysis, glass electrode, and hydrogen electrode methods and found very good agreement between the two electrometric methods. Comparing the dialysis and the glass electrode methods they showed that the correction necessary to make the colorimetric readings correspond to the electrometric readings varied from -0.25 pH to $+0.25$ pH. These variations are not only much greater than ours, but they differ also in that our colorimetric readings were practically never lower than electrometric readings. We have only twice observed a small negative correction. As the average of their variations is about zero they draw the conclusion that the dialysis method is accurate to 0.02 pH.

Although there are data in the literature showing variations in the corrections of the colorimetric readings they have aroused little concern on the part of those using the colorimetric methods. Conclusions have been drawn from colorimetric data without consideration of the possibility of variations in the correction. In Table II there are collected, from various sources, data which not only indicate the variability of colorimetric corrections but also point out that the corrections are variable in other species as well as the dog.

Of the colorimetric methods studied, the Hastings and Sendroy modification of Cullen's dilution method yielded the smallest corrections and minimum variations in the corrections. It would

TABLE II.
Variations in Colorimetric Corrections.

Author.	Source.	Dilution method.		Dialysis method.	
		Cullen, $pH_{20} - pH_{38}^*$.	Hastings, $pH_{38} - pH_{38}^*$.	$pH_{20} - pH_{38}^*$.	$pH_{38} - pH_{38}^*$.
Conway-Verney and Bayliss (1923-24).	Sheep.				
Chambers and Kleinschmidt (1923).	Human (normal). (Clinical cases.) †				
Hastings and Sendroy (1924).			0.02-0.04 (10)		0.02-0.05 (1)*
Marrack and Smith (1924).	Human (clinical cases).	0.20-0.26 (19)			
Austin, Stadie, and Robinson (1925).	Dog.	0.20-0.47 (21)	0.00-0.17 (28)	0.00-0.20 (12)	
	Sheep.	0.06-0.27 (7)	0.00-0.06 (11)	0.10-0.31 (31)	
	Rat.	0.16-0.18 (2)	0.02-0.06 (2)		
	Human (clinical cases).	0.19-0.51 (8)	0.01-0.20 (11)		
	" "	0.24-0.35 (18)	0.01-0.12 (9)*		
Cullen, Keeler, and Robinson (1925).					
Earle and Cullen (1928).	Human (normal).		0.06-0.09 (11)*		
Bennett (1926).	Dog.	0.21-0.46 (34)	0.15-0.22 (5)		
Present report.	"	0.17-0.50 (36)	0.00-0.18 (38)	0.00-0.41 (44)	0.02-0.32 (16)

Figures in parentheses indicate number of different bloods studied.

* Both colorimetric and electrometric determinations run at the same temperature but other than 38°, usually 20°.

† Bloods studied were horse (5), dog (1), rat (1), normal human (2).

appear that the procedure is probably suitable for much work where errors of 0.10 pH are not objectionable, but for accurate studies where small variations are of importance it is obviously untrustworthy.

After this paper was sent to the publishers a statistical study on the same subject appeared by Myers and Muntwyler (1928). They report comparisons of results obtained by using the Cullen, Hastings, and hydrogen electrode methods on 103 samples of human plasma from restricted groups of patients and ten samples of dog plasma. They find that, when a constant Cullen correction of 0.22 pH is subtracted to correct the colorimetric readings obtained by Cullen's method, 59 per cent of the colorimetric values were within ± 0.02 pH, 74 per cent within ± 0.03 pH, and 85 per cent within ± 0.04 pH of the electrometric values. The remaining 15 per cent of the colorimetric readings differed from the electrometric readings by as little as 0.14 and as much as 0.30 pH so that the use of the constant Cullen correction with this group would yield results having considerable error. The data obtained by the Hastings method showed similar variations, no correction being used. The conclusion is drawn that "very good agreement" was obtained. Such a conclusion agrees with the observation of Bayliss, Kerridge, and Verney that the average of the differences between the Dale and Evans method and the glass electrode method equalled zero, though the differences ranged from $+0.25$ to -0.25 pH.

These conclusions are in such sharp contrast with those drawn by Bennett, Austin, Stadie, and Robinson, and the present writer that some explanation is necessary to clarify the situation. There is no indication that there are serious errors in the data of any of these investigations. The different conclusions are probably due to differences in the points of view of the investigators.

The original work of Cullen as well as the later contributions of Hastings, Bayliss, Kerridge, and Verney, Earle and Cullen, and Myers and Muntwyler all show that for a particular procedure the colorimetric corrections group themselves around an average value. For statistical studies, at least for bloods from certain groups of individuals, the use of the average correction will yield a high percentage of results which will agree sufficiently closely with the hydrogen electrode values. The recent paper of Earle and Cullen confirms Cullen's correction for normal humans. The

data of Myers and Muntwyler show that certain pathological conditions yield a high percentage of bloods having the same average correction as normal bloods. The studies of Austin, Stadie, and Robinson and of Hastings demonstrate that in certain pathological conditions the correction varies from the normal so much that erroneous information would be obtained by subtracting a normal average correction value from the colorimetric readings. In view of these facts care must be taken in drawing conclusions from individual colorimetric determinations on bloods from pathological human cases until much more complete studies have been made and the variation in the colorimetric corrections more completely determined.

Dog serum has a higher colorimetric correction than human serum; that is, the difference between the colorimetric and electrometric pH values of dog serum is greater than that of normal human serum. It is not surprising therefore that variations in the correction are more frequently encountered. Undoubtedly the average Cullen correction for a large series of normal dogs would not vary far from the value of 0.35 pH originally determined by Cullen. But in the paper here reported as well as in other publications the striking variations in the colorimetric corrections which may occur even in blood from a single animal when subjected to a heavy hemorrhage emphasize the need for care in using a colorimetric method for determining variations in pH of blood in an experimental animal. Even Myers and Muntwyler report, in a series of six sera obtained from one dog, Cullen corrections varying from 0.27 to 0.43.

SUMMARY.

Comparisons were made of the hydrogen ion concentration of dog sera as determined by colorimetric methods and the hydrogen electrode method. Cullen's, Hastings' modification of Cullen's, and Dale and Evans' colorimetric methods were used. The differences between the colorimetric readings and electrometric readings (designated as colorimetric corrections) were not constant. The corrections for sera from an individual animal after severe hemorrhage showed extreme variations. The corrections are so variable that the colorimetric methods cannot be used if accurate comparisons of individual determinations are to be made.

A survey of the literature indicates that colorimetric methods may be used for statistical studies on human blood from normal and certain groups of pathological individuals with average correction values. At the present time it is doubtful if colorimetric methods should be used indiscriminately on all varieties of sera without adequate checking with the hydrogen electrode. For a comparison of individual determinations of either human or dog bloods colorimetric methods should not be used.

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THE MECHANISM OF EPINEPHRINE ACTION.

I. THE INFLUENCE OF EPINEPHRINE ON THE CARBOHYDRATE METABOLISM OF FASTING RATS, WITH A NOTE ON NEW FORMATION OF CARBOHYDRATES.

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Dudley and Marrian (1) were the first to show that liver glycogen disappears in fasting animals when insulin is injected. Cannon, McIver, and Bliss (2) found that insulin hypoglycemia leads to an increased discharge of epinephrine. Furthermore, there was general agreement that epinephrine mobilizes liver glycogen. By linking these three facts together, a strong case was made out for the assumption that the disappearance of liver glycogen in insulinized animals was due to the discharge of epinephrine. This assumption is contradicted by the recent observation (3) that insulin mobilizes liver glycogen in adrenalectomized animals; that is, in animals with an abolished or at least strongly reduced epinephrine secretion. A decrease in liver glycogen in adrenalectomized rats following insulin injections has been found independently by Artundo (4).

The results of Cannon and his collaborators and of other investigators are accepted as evidence that insulin evokes an increased discharge of epinephrine, while the observations on adrenalectomized animals referred to above are taken as evidence that the disappearance of liver glycogen following insulin injections is not connected with this increased release of epinephrine. In view of this it was difficult to see how the antagonistic action of insulin and epinephrine on blood sugar was brought about. The following question presented itself. If insulin alone is capable of a rapid glycogen mobilization in the liver, and if epinephrine has its chief point of attack on liver glycogen, how does epinephrine antagonize the insulin hypoglycemia? There

was the possibility that epinephrine, whether released by the adrenals or injected, speeded up the mobilization of liver glycogen. However, there was no marked difference in the rate of disappearance of liver glycogen in normal and adrenalectomized animals, when insulin was injected. This made it unlikely that the antagonistic action between insulin and epinephrine occurred in the liver; indeed, it seemed more probable that the chief antagonistic action between these two hormones took place outside of the liver. A detailed study of the literature gave no definite clue as to the influence of epinephrine on the carbohydrate metabolism of the peripheral tissues. It appeared that the hepatic action of epinephrine had been overemphasized, with the result that an extrahepatic point of attack of this hormone was not taken into consideration. This made it desirable to supplement our knowledge of epinephrine in this respect. As in the case of

TABLE I.
Glycogen Content in Mg. per 100 Gm. of Rat.

	Liver.	Rest of body.	Total.
24 hr. fasting.....	7 \pm 2	136 \pm 10	143 \pm 11 (Average of 16.)
48 " "	10 \pm 2	111 \pm 13	121 \pm 13 (" " 21.)
Difference.....	+3	-25	-22

insulin, the most promising approach in the investigation of an extrahepatic action, seemed by way of a sugar balance. Experiments involving a sugar balance were carried out on sugar-fed, on rats in the postabsorptive state, and on 24 hour fasting rats. They were made in the chronological order just given, but for reasons of greater ease of presentation, the order in which these experiments are published is reversed.

Note on the New Formation of Carbohydrates.

The following observation involves the problem of new formation of carbohydrates. It was found that the glycogen content of rats undergoes only a slight diminution between the 24th and 48th hour of fasting. This is shown in the summary in Table I, which was calculated from previous experiments (5-7), including the present series of glycogen determinations in Table II. The average deviations from the mean are given in Table I in order

to illustrate the extent of individual variation. It will be noted that there is a diminution of only 22 mg. of glycogen between the 24th and the 48th hour of fasting, in spite of the fact that the animals perform muscular work during that time. It seems clear that 22 mg. of glycogen can provide only a small fraction of the lactic acid needed for the performance of muscular work, and it is therefore evident that much more than 22 mg. of glycogen is split into lactic acid during a period of 24 hours. Nevertheless, the glycogen content of the rats remains practically constant. This may have two causes. Either glycogen is not used up, because the oxidation of fat furnishes the energy for the reconversion of lactic acid into glycogen, or glycogen is formed from non-carbohydrate material as fast as it is used up. In the latter case sugar formed from non-carbohydrate sources in the liver must be transported to the muscles by means of the blood stream.

The view that fat oxidation may furnish the energy for the reconversion of lactic acid is held by Lusk (8). Krogh and Lindhard (9), in their study of muscular work, came to the conclusion that the R.Q., as determined, is always a mixture of anabolic and catabolic processes. With an R.Q. above 0.9 sugar is converted in part into fat; with an R.Q. below 0.8 the reverse process takes place. A number of investigators came to regard the conversion of fat into sugar as a proved fact, even though the Danish authors stated expressly that they were proposing only a working hypothesis. According to this hypothesis, part of the fat undergoing oxidation passes through a carbohydrate stage before it appears as CO_2 and H_2O . There can be no objection to this view, because the sugar stage in the breakdown of the fat molecule is conceived as transitory and is regarded as taking place in the same cell in which the oxidation of fat is completed. A transportation of the sugar formed from fat, from one part of the body to another, is not postulated. If such a transportation would take place, the sugar formed from fat should give rise to the excretion of sugar under special conditions. The view of Krogh and Lindhard is, therefore, not necessarily opposed to the fact that the catabolism of fat does not lead to the excretion of sugar in the diabetic organism.

The constancy of the glycogen of fasting rats is best explained either by the view of Lusk, or by the conception of Krogh and

Lindhard. Those, who believe that the energy for the reconversion of lactic acid into glycogen can come from the oxidation of carbohydrates only, will favor the latter view. The constancy of the glycogen is less satisfactorily explained by the assumption of Chaikoff and Macleod (10). These authors believe that the liver converts fat into sugar and has therefore an R.Q. of about 0.3. The sugar formed from fat in the liver is carried to the muscles where it undergoes oxidation with an R.Q. of unity. The R.Q. of the whole animal is then the algebraic sum of the R.Q.'s of the liver and muscles. Numerous measurements have shown that the R.Q. of 24 and 48 hour fasting rats is invariably in the neighborhood of 0.71. In order to explain these R.Q.'s by Macleod's theory, one has to assume that the new formation of sugar in the liver and the oxidation of this sugar in the muscles are exactly synchronized. If at any time the sugar formed from fat were to accumulate in the body, the R.Q. would fall markedly below 0.71, which has not been observed. The same consideration applies to the diabetic R.Q. The sugar formed from fat in the diabetic organism, instead of being excreted in the urine, must be carried to the muscles and must be oxidized as fast as it is formed. If any sugar formed from fat would escape in the urine, the R.Q. would fall to a low level. Lusk (11) writes that the establishment of the diabetic quotient at a level of 0.69 carries the refutation of the idea that fat may be converted into sugar. The amino acids, which are convertible into glucose in the liver of the diabetic animal, appear as sugar in the urine. It is difficult to see why the sugar formed from fat in the liver, as postulated by Macleod's theory, should not have the same fate.

It is proposed to make a distinction between sugar formation from non-carbohydrate sources as an intermediary step of oxidation, and gluconeogenesis (or new formation of carbohydrates). The former case may be called intermediary sugar formation, denoting the molecule from which the sugar is formed (fat, protein, *etc.*). The latter term should be reserved for cases where the sugar formed from non-carbohydrate sources becomes stabilized and is, therefore, capable of becoming blood sugar and of being transported to other parts of the body. The conception of Krogh and Lindhard affords an example for intermediary sugar formation from fat. An example for gluconeogenesis is sugar

formation from protein in the diabetic animal. The authors believe that it is permissible to speak of intermediary sugar formation from fat in the same way as one discusses various possibilities for intermediary stages of carbohydrate and protein oxidation. However, gluconeogenesis from fat is still awaiting experimental verification.

Experiments with Epinephrine.

The foregoing discussion may serve as a background for the changes observed in liver and body glycogen and in respiratory metabolism in 24 hour fasting rats, following the subcutaneous injection of epinephrine. Rats of 125 to 145 gm. of body weight were fasted for 21 hours. A metabolism fore period of 3 hours

TABLE II.
Glycogen Content of 24 Hour Fasting Rats.

Body weight.	Liver weight per 100 gm. of body weight.	Glycogen content per 100 gm. of body weight.			Blood sugar.
		In liver.	In other tissues.	Total.	
gm.	gm.	mg.	mg.	mg.	mg.
123	3.42	3.0	123.0	126	85
132	3.58	4.2	129.8	134	93
130	3.62	9.9	144.1	154	87
141	3.48	7.1	114.9	122	81
	3.52	6.0	128.0	134	87

was made, 0.02 mg. of epinephrine per 100 gm. of body weight was injected, and immediately a second metabolism period of 3 hours was started. The animals were killed 3 hours after the injection, and the glycogen in the liver and the rest of the body was determined in the usual manner. The amount of glycogen present in liver and body at the time of the epinephrine injection was determined on a series of control rats (Table II).

In the experiments with epinephrine in Table III the average R.Q. of the fore period was 0.715, corresponding to a non-protein R.Q. of 0.704. This R.Q. allows for no oxidation of preformed carbohydrates. The validity of this R.Q. is established by the observation recorded in Table I, that there is a disappearance of only 22 mg. of glycogen between the 24th and the 48th hour of fasting. The average

R.Q. of the epinephrine period remained unchanged as compared with the fore period, while the O_2 consumption rose in every experiment, corresponding to an average increase in calorie production of 17.3 per cent. Epinephrine produced an increase in heat production in 24 hour fasting rats at the expense of fat oxidation,

TABLE III.

Influence of Epinephrine on Carbohydrate Metabolism of 24 Hour Fasting Rats.

Average body weight 135.0 ± 7 gm. Values calculated per 100 gm. of body weight per 3 hours.

Fore period (3 hrs.).			Epinephrine period (3 hrs.).								
O_2	R.Q.	Total calories.	O_2	R.Q.	Glycogen.			Urine N.	Blood sugar.	Total calories.	Increase in calories over basal.
					In liver.	In rest of body.	Total.				
gm.			gm.		mg.	mg.	mg.	mg.	mg.		per cent
0.660	0.710	2.27	0.799	0.722	28.4	58.4	86.8	8.81	105	2.61	15.0
0.768	0.734	2.50	0.844	0.717	50.2	81.9	132.1	7.00	105	2.75	10.0
0.632	0.699	2.05	0.768	0.717	45.9	65.5	111.4	8.40	137	2.50	21.9
0.654	0.720	2.13	0.782	0.718	43.1	76.5	119.6	7.96	104	2.55	19.7
0.648	0.711	2.10	0.770	0.714	32.6	62.8	95.4	11.76	100	2.50	19.0
0.703	0.714	2.28	0.839	0.701	51.8	77.2	129.0	11.37	110	2.72	19.5
0.677	0.715	2.22	0.800	0.715	42.0	71.0	113.0	9.22	110	2.60	17.3

TABLE IV.

Average Glycogen Content in Mg. per 100 Gm. of Rat.

Calculated from Tables II and III.

	Liver.	Rest of body.	Total.
Controls.....	6 ± 2	128 ± 9	134 ± 10
3 hrs. after epinephrine.....	42 ± 8	71 ± 8	113 ± 15
Difference.....	+36	-57	-21

since an increased nitrogen elimination in the urine was not observed. This indicates that the calorogenic action of epinephrine is not necessarily connected with an increased combustion of carbohydrates.

There was a slight but unmistakable rise in blood sugar. The control rats in Table II showed an average of 87 mg. per cent,

while 3 hours after the epinephrine injection the blood sugar averaged 110 mg. per cent. The R.Q. of the fore period and of the 3 hour period during which epinephrine acted, showed no indication of carbohydrate oxidation. Nevertheless, profound changes were produced in the distribution of glycogen by the epinephrine injections. This is the more remarkable because these changes occurred in animals which were in a state of glycogen equilibrium. It will be seen from a comparison of the glycogen values in Tables II and III that epinephrine leads to a marked increase in liver glycogen and to a diminution of body glycogen in every experiment. On an average, the glycogen in the rest of the body is found to have decreased a little more than the liver glycogen has increased (-57 against $+36$ mg.). This is shown in the summary in Table IV, which was calculated from Tables II and III. The liver glycogen which had accumulated during the 3 hours of epinephrine action, was found to have disappeared again when the rats were killed 16 hours after the injection.

An increase in liver glycogen after epinephrine has been observed previously by Loeper and Crouzon (12), by Pollak (13), and by Kuriyama (14). None of these authors determined the glycogen so soon after the epinephrine injections as in the present experiments. Pollak made rabbits glycogen-free by fasting and strychnine poisoning. After daily injections of epinephrine, a considerable amount of liver glycogen (up to 4.5 per cent) was formed, in spite of the continued fasting. Kuriyama found that the livers of rabbits which were fasted and submitted to daily epinephrine injections, contained a much larger amount of glycogen than those of merely fasted animals. On an average, the muscle glycogen of the injected rabbits was lower than that of the control rabbits. The interval between the last epinephrine injection and the glycogen determination varied between 7 and 24 hours. A single injection of epinephrine into fasted rabbits also caused an increase in liver glycogen. Recently, Markowitz (15) observed that repeated epinephrine injections into fasted, strychnine-treated rabbits lead to an accumulation of liver glycogen. Since a complete carbohydrate balance was not made, this author arrived at the conclusion that the newly formed glycogen in the liver was derived from fat.

The present experiments reveal that muscle glycogen is another

possible source for the newly formed liver glycogen. It is now definitely known, especially from the work of Mann and Magath (16) on hepatectomized dogs, that muscle glycogen is not a direct source of blood sugar after epinephrine injections, or under any other conditions. When muscle glycogen is split, the presence of the glycolytic ferment causes an immediate change of the split products into lactic acid (Lohmann (17)). The course of events in 24 hour fasting rats would then be that epinephrine mobilizes muscle glycogen and that lactic acid enters the blood stream and is carried to the liver where it is deposited as glycogen. If the disappearing muscle glycogen (57 mg.) were oxidized, the R.Q. should be 0.74, while the R.Q. actually observed was 0.715.

Before considering this explanation further, it was necessary to determine whether lactic acid was able to form liver glycogen. *d*-Lactic acid in the free state or as sodium salt, as well as *r*-sodium lactate, when fed by stomach tube to 24 hour fasting rats, gave rise to the formation of considerable amounts of liver glycogen (18). Sodium lactate, when injected subcutaneously, was far less effective in forming liver glycogen. A detailed report of these experiments will be presented in a later paper. Abramson, Eggleton, and Eggleton (19) reported recently that *r*-sodium lactate, when injected intravenously, does not form liver glycogen. It would seem that their experimental procedure was not favorable for the detection of glycogen formation from lactic acid.

With the demonstration that lactic acid forms liver glycogen, one possible objection to the explanation here proposed has been eliminated. An older observation gains special significance in the light of the present work. It was found that epinephrine injections lead to a marked increase in the blood lactic acid of rabbits and cats (20). Tolstoi, Loebel, Levine, and Richardson (21) observed an increase in blood lactic acid in men after epinephrine injections. An increase was also found, to a lesser extent, in the rats used for the present experiments.

There is the possibility that the liver glycogen accumulating during epinephrine action is derived from non-carbohydrate sources, in other words, that one is dealing with gluconeogenesis according to the definition given in the preceding section. If this were the case, one would have to assume that the glycogen disappearing from the rest of the body is oxidized. The simultaneous

occurrence of the two processes (anabolic and catabolic) would also result in the R.Q. actually observed. The average nitrogen elimination in the 3 hour epinephrine period was 9.2 mg. If one assumes a conversion into glucose corresponding to a D:N ratio of 3.65, 34 mg. of glucose could have been derived from the catabolism of protein. The increase in liver glycogen of 36 mg. is of the same magnitude. This indicates that it is unnecessary to assume gluconeogenesis from fat in order to explain the increase in liver glycogen after epinephrine injections. We believe that gluconeogenesis does not explain the results, because an increase in liver glycogen was also observed in rats in the postabsorptive state in which gluconeogenesis should be in abeyance on account of previous carbohydrate feeding and an R.Q. above 0.8. The increase in blood lactic acid is also in favor of the first explanation.

The experiments on 24 hour fasting rats recorded in this paper show in a definite way that epinephrine influences the carbohydrate metabolism of the peripheral tissues, since it leads to the disappearance of muscle glycogen. The experimental demonstration of glycogen formation in the liver from lactic acid supports the conclusion that glycogen mobilized in the muscles is converted into liver glycogen with lactic acid as an intermediary stage. Muscle glycogen is therefore an indirect source of blood sugar if the liver is present and if there is an escape of lactic acid from the muscles. Epinephrine injections, violent exercise, strychnine and insulin convulsions, asphyxia, decerebration, and a variety of other conditions lead to an escape of lactic acid from the muscle. The loss of muscle glycogen incurred under these different conditions is repaid, in part at least, by the liver in terms of glucose. It remains an established fact that the liver is the only direct source of blood sugar in the body. In the absence of the liver, muscle glycogen is unable to contribute sugar to the blood, because lactic acid cannot be converted into liver glycogen.

SUMMARY AND CONCLUSIONS.

1. Between the 24th and 48th hour of fasting, the glycogen content of rats diminishes by only 22 mg. This constancy of the glycogen during fasting is discussed from the standpoint of the new formation of carbohydrates. It is proposed to make a

sharp distinction between carbohydrate formation from protein or fat as an intermediary step in the complete oxidation of these molecules and gluconeogenesis. The latter term should be reserved for cases where the sugar formed from non-carbohydrate sources becomes stabilized.

2. The R.Q. of 24 hour fasting rats of 0.715 remained unchanged when epinephrine was injected. The O_2 consumption rose in each experiment, corresponding to an average increase in heat production of 17.3 per cent.

3. 3 hours after the subcutaneous injection of epinephrine (0.02 mg. per 100 gm. of rat), the body glycogen (mainly muscle glycogen) diminished on an average by 57 mg., while the liver glycogen increased by 36 mg.

4. The increase in liver glycogen after epinephrine injections is explained by the conversion of muscle glycogen into liver glycogen with lactic acid as an intermediary stage. This explanation is supported by the fact that the oral administration of lactic acid has been found to lead to the deposition of liver glycogen. Another possibility which has been considered is the conversion of protein into liver glycogen, with a simultaneous oxidation of the glycogen disappearing in the muscles. The combination of the two processes (anabolic and catabolic) would also result in the R.Q. actually observed.

5. Accepting the first explanation as the better supported one, it follows that muscle glycogen is an indirect source of blood sugar, if the liver is present and if there is an escape of lactic acid from the muscles.

6. Epinephrine influences the carbohydrate metabolism of the peripheral tissues, since it leads to the disappearance of muscle glycogen.

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THE MECHANISM OF EPINEPHRINE ACTION.

II. THE INFLUENCE OF EPINEPHRINE AND INSULIN ON THE CARBOHYDRATE METABOLISM OF RATS IN THE POSTABSORPTIVE STATE.*

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The experiments on 24 hour fasting rats recorded in the preceding paper (1) have shown that epinephrine injections lead to a disappearance of muscle glycogen and to an accumulation of liver glycogen. The glycogen mobilized in the muscles was apparently not oxidized, and it was suggested that muscle glycogen was converted into liver glycogen with lactic acid as an intermediary stage. Before describing the present experiments it may be advisable to examine the evidence so far accumulated as to the behavior of liver and muscle glycogen in animals in the postabsorptive state after epinephrine injections.

HISTORICAL.

Shortly after Blum (2) had discovered in 1901 that injections of adrenalin cause glycosuria, several authors attempted to investigate the influence of such injections on the glycogen stores of the body. In some of these investigations, methods were used which were shown later to account for only fractions of the glycogen present (Doyon and Kareff (3), Doyon, Morel, and Kareff (4)). These will not be considered here in detail. In 1905 Bierry and Gatin-Gruzewska (5), using Pfüger's method, determined the liver glycogen of four dogs, 4 to 5 hours after an adrenalin injection. The values found varied from 0.16 to 1.5 per cent. Control values are not recorded, which is a serious objection in view of the results obtained in another paper of Gatin-Gruzewska (6). Here adrenalin was given to 24 hour fasting rabbits and the animals were killed by bleeding 32 to 40 hours after the injection. Neither muscle nor liver glycogen was found, but the control animals also contained no liver glycogen and only 0.05 per cent

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muscle glycogen. Paton (7) and Drummond and Paton (3) concluded from experiments in which liver glycogen was determined in three injected and two control rabbits that it decreases after the injection of one large dose but not when adrenalin is given repeatedly for several days. Agadschanian (9) working with the old water extraction method and with Pfüger's method but weighing the glycogen (both not quantitative procedures), killed rabbits 36 hours after the injection of 1.0 to 1.5 mg. of adrenalin. The values for liver glycogen of his injected rabbits were 0, 0, 0.09, 0.11, for the controls 0.06, 0.6, 0.27 per cent. No muscle glycogen was found in the injected and only 0.05, trace, 0.025, and 0.1 per cent in the control rabbits. Doyon and Gautier (10) took liver samples from the same dog before and 26 to 45 minutes after the injection of large doses of epinephrine into a mesenteric vein. In all cases there was a decrease in liver glycogen. (8.05 to 3.66, 8.04 to 5.05, 7.9 to 5.7, 5.48 to 3.77, 5.38 to 3.74). Control experiments without injection were not performed.

This is the principal evidence to be found in the literature on a decrease in liver and muscle glycogen after epinephrine injections. The objections to these papers are obvious. In view of the great variability of liver and muscle glycogen of the species used, a sufficient number of animals has not been used by any of the authors. The extremely low glycogen values of most of the controls cast doubt on the analytical technique of several workers. Postmortem glycogenolysis was not sufficiently recognized at that time and probably no precautions were taken to avoid it. Bleeding the animals to death is also an unsuitable procedure because it leads to premortal glycogenolysis. Where liver glycogen was determined on the same animal before and after the injection, narcosis and laparotomy had to be resorted to, procedures which in themselves lower the liver glycogen. In many instances the animals were killed 1 or even 2 days after the injection at a time when epinephrine had long ceased to act. In spite of these shortcomings this work has been accepted as evidence that epinephrine causes a decrease in liver glycogen and the authors mentioned are usually quoted together in text-books and reviews as having proved this point (*cf.* the extensive review by Trendelenburg (11)). When it was found that muscle glycogen is not a direct source of blood sugar¹ (Velich (13), Vandeput (14), Falta and Priestley (15)), the claim of the above authors that epinephrine leads to a decrease in muscle glycogen was disregarded. In this way the hepatic action of epinephrine was given a predominant rôle, while an extrahepatic point of attack was gradually left out of consideration. The high blood sugar was taken as further evidence that the liver glycogen diminishes after epinephrine, since it seemed inconceivable that a hyperglycemia could develop without a decrease in liver glycogen.

Another group of authors (Loeper and Crouzon (16), Pollak (17), Kuriyama (18)) found that liver glycogen increases in fasting animals after epinephrine injections, in spite of a marked hyperglycemia and usually also

¹ It is only since the experiments of Mann (12) that this may be regarded as a well established fact.

glycosuria. A larger number of animals was used in the work of the last two authors, and there was little variability in the controls on account of the longer fasting period. Since no satisfactory explanation could be offered for this paradoxical behavior of liver glycogen, the results of Pollak and Kuriyama did not lead to a reconsideration of the mechanism of epinephrine action. Recently Junkersdorf and Schöler (19) and Junkersdorf and Török (20) found higher liver glycogen in well fed dogs receiving epinephrine than in suitable controls. In underfed dogs rather the opposite tended to be the case. Under both conditions the muscle glycogen of the injected dogs was markedly lower. Markowitz (21) also observed an increase in liver glycogen in fasting rabbits receiving daily epinephrine injections.

In summarizing one may say that it has not been proved definitely that liver glycogen diminishes after epinephrine injections, nor are there any data available which indicate how much liver glycogen disappears. Future work will probably show that the liver glycogen diminishes under certain conditions, especially if there is strong glycosuria, but this is still awaiting experimental verification. On the other hand, liver glycogen has been found to increase after epinephrine injections. A decrease in muscle glycogen has been claimed by all workers who analyzed for it, though much of the work has not been sufficiently controlled.

It was rather surprising to find, by going back to the sources, that these contradictory results form the basis for the present explanation of epinephrine hyperglycemia and glycosuria. According to this explanation hyperglycemia and glycosuria develop because a larger amount of sugar is given off by the liver than can be cared for by the tissues. It has apparently not been considered that there is, as a rule, not enough liver glycogen present to supply the body for hours with amounts of sugar greater than the normal tolerance limit of the tissues. The fact that liver glycogen is sometimes higher after epinephrine injections should have cast doubt on the assumption that the only cause of the hyperglycemia consists in a mobilization of liver glycogen. By assigning a purpose to the epinephrine hyperglycemia, namely, to facilitate utilization of blood sugar in the muscles, attention has been distracted further from the cause of the hyperglycemia. In the present paper a new conception of the mechanism of epinephrine action is advanced and it is shown that there are other factors involved in the production of hyperglycemia besides mobilization of liver glycogen.

In the present experiments the animals were rich in glycogen, while the experiments on 24 hour fasting rats (1) were made on animals with low carbohydrate reserves. There is also this differ-

ence between the experiments on 24 hour fasting rats and rats in the postabsorptive state. In the former animals the glycogen is in a stationary condition at the time the epinephrine injections are made; in the latter animals glycogen disappears even if no epinephrine is injected. As will be shown later, epinephrine greatly accelerates the disappearance of muscle glycogen in rats in the postabsorptive state; at the same time it leads to an accumulation of liver glycogen. The changes in glycogen are therefore of the same type as in 24 hour fasting rats.

A method has been worked out which permits the calculation of the amounts of glycogen disappearing from liver and muscles of rats in the postabsorptive state. This method is described in detail below. The simultaneous determination of sugar oxidation makes it possible to strike a sugar balance, because one is in a position to judge how much of the disappearing glycogen is accounted for by sugar oxidation.

The following three series of experiments have been made on rats in the postabsorptive state: one series on control rats which received an injection of saline; a second series with the subcutaneous injection of 0.02 mg. of epinephrine per 100 gm. of rat which was also the dose employed in the experiments on 24 hour fasting rats; finally, a third series with the injection of 0.75 units of insulin per 100 gm. of rat. In each case the animals were killed 3 hours after the injection.

Method of Determining the Disappearance of Glycogen in Rats in the Postabsorptive State.

Male rats of 130 to 160 gm. of body weight were fasted for 24 hours. The urine of the last 17 hours was collected for nitrogen determinations. 2.5 cc. of a glucose solution, containing 1.065 gm. of glucose per 100 gm. of body weight, were fed by stomach tube. This is the average amount of glucose absorbed in 4 hours by 24 hour fasting rats (22). The respiratory metabolism was determined during the 4 hours of glucose absorption. Since the amount of glucose absorbed and oxidized during these 4 hours is known, one can calculate how much glycogen is being deposited during that time. (This calculation is made on the basis of previous experiments (22) in which the average amount of absorbed glucose accounted for by sugar oxidation and glycogen formation

TABLE I.

Influence of Epinephrine and Insulin on Sugar Oxidation and Glycogen Content of Rats in the Postabsorptive State.

Values calculated per 100 gm. of body weight.

Glucose absorption period (4 hrs.).				Postabsorptive period (3 hrs.)*							
O ₂	R.Q.	Glucose absorbed.	Glucose oxidized.	O ₂	R.Q.	Glucose oxidized.	Glycogen.			Urine N per hr.	Blood sugar.
							In liver.	In rest of body.	Total.		
Controls, saline injection.											
gm.		gm.	gm.	gm.		gm.	gm.	gm.	gm.	mg.	mg.
0.795	0.857	1.087	0.356	0.567	0.859	0.255	0.210	0.345	0.555	2.85	127
0.899	0.844	1.042	0.367	0.628	0.801	0.169	0.154	0.254	0.408	2.83	99
0.842	0.887	1.067	0.458	0.635	0.847	0.263	0.164	0.243	0.407	2.95	102
0.903	0.885	1.064	0.488	0.659	0.809	0.195	0.157	0.215	0.372	2.82	123
0.860	0.868	1.065	0.417	0.622	0.829	0.220	0.171	0.264	0.435	2.86	113
Epinephrine injection.											
0.836	0.832	1.034	0.304	0.765	0.844	0.312	0.226	0.139	0.365	3.25	176
0.846	0.859	1.066	0.383	0.749	0.821	0.254	0.269	0.139	0.408	2.88	191
0.913	0.870	1.080	0.448	0.736	0.825	0.255	0.226	0.135	0.361	3.11	148
0.893	0.879	1.077	0.463	0.712	0.831	0.261	0.257	0.124	0.381	2.87	158
0.961	0.875	1.022	0.482	0.771	0.811	0.231	0.239	0.113	0.352	3.64	196†
0.889	0.863	1.056	0.416	0.747	0.826	0.263	0.244	0.130	0.374	3.15	174
Insulin injection.											
0.782	0.838	1.069	0.293	0.589	0.932	0.400	0.129	0.334	0.463	3.30	53
0.838	0.860	1.022	0.380	0.613	0.946	0.448	0.050	0.249	0.299	2.73	72
0.936	0.862	1.076	0.438	0.616	0.945	0.450	0.075	0.194	0.269	2.76	69
0.794	0.902	1.046	0.468	0.586	0.951	0.437	0.084	0.223	0.307	2.87	81
0.837	0.865	1.053	0.395	0.601	0.943	0.434	0.085	0.250	0.335	2.92	69

* Injections were made at the beginning of the postabsorptive period.

† Double dose of epinephrine (0.04 mg. per 100 gm.) injected. 10 mg. of sugar excreted in urine.

in liver and the rest of the body was 86.5 per cent.) 4 hours after the glucose feeding the animals were in the postabsorptive state. Then the injections of saline, epinephrine, or insulin were made. The respiratory metabolism was determined for 3 hours after the injection. After this time the animals were killed and the glycogen content of the liver and the rest of the body was determined in the usual manner. Since one knows how much glycogen is present at the time of injection as well as 3 hours after the injection, the former by computation and the latter by direct determination, one can calculate how much glycogen disappears during 3 postabsorption hours, and one can compare this with the amount of glucose that has been oxidized during that time.

The following example (Rat 4 of the control series in Table I) illustrates how this calculation is carried out.

4 Hour Glucose Absorption Period.

Weight of rat 132.5 gm. Glucose fed by stomach tube 1.41 gm. Per 100 gm. of body weight per 4 hours: glucose absorbed 1.064 gm.; glucose oxidized 0.488 gm. In Table XI of a previous paper (22) an average of 86.5 per cent of the absorbed glucose is accounted for. The quotient

$$\frac{\text{glycogen formed in liver}}{\text{total glycogen formed}}$$

is 0.422 and the quotient $\frac{\text{glycogen formed in the rest of the body}}{\text{total glycogen formed}}$ is 0.577.

On this basis one finds:

$$\frac{1.064 \times 86.5}{100} = 0.920 \text{ gm. of absorbed glucose accounted for.}$$

$$0.920 - 0.488 = 0.432 \text{ gm. of glucose converted into glycogen.}$$

$$0.432 \times 0.422 = 0.182 \text{ gm. of glucose converted into liver glycogen.}$$

$$0.432 \times 0.577 = 0.250 \text{ gm. of glucose converted into body glycogen.}$$

In order to find the total amount of glycogen present at the time of injection, add preformed glycogen of 24 hour fasting rats (22) (8 mg. for liver and 140 mg. for the rest of the body).

$$0.182 + 0.008 = 0.190 \text{ gm. of glycogen present in the liver.}$$

$$0.250 + 0.140 = 0.390 \text{ gm. of glycogen present in the rest of the body.}$$

3 Hour Period after the Injection. (Postabsorptive Period).

The glycogen determinations 3 hours after the injection, calculated per 100 gm. of body weight, gave the following values: liver 0.157, body 0.215 gm.

Hence, $0.190 - 0.157 = 0.033$ gm. of glycogen disappeared from the liver.

$0.390 - 0.215 = 0.175$ gm. of glycogen disappeared from the rest of the body.

Owing to the difference in blood sugar concentration at the beginning (158 mg. per cent) and at the end of the 3 hour period (123 mg. per cent), there is also a disappearance of glucose from the blood and body fluids. It is assumed that the blood is in equilibrium with 50 per cent of the body weight. Therefore: $\frac{158}{2} - \frac{123}{2} = 18$ mg. of glucose disappeared from the blood and body fluids.

The total amount of glucose which disappeared during the 3 hours after the injection was $0.033 + 0.175 + 0.018 = 0.226$ gm. The glucose oxidized

TABLE II.
Glycogen Content of Liver and Body at Time of Injections.
Calculated from Table I.

	Liver.	Rest of body.	Total.
	mg.	mg.	mg.
Controls, saline injection.	254	478	732
	233	449	682
	204	409	613
	190	390	580
	220	432	652
Epinephrine injection.	257	481	738
	235	452	687
	213	421	634
	206	411	617
	178	372	550
	218	427	645
Insulin injection.	275	505	780
	221	431	652
	216	425	641
	192	393	585
	226	438	664

during that time, as calculated from the non-protein R.Q., was 0.195 gm. Hence, 86.4 per cent of the calculated loss of glucose has been accounted for by oxidation.

Results.

Table I contains the data from which the calculations in the manner just described have been made. The amounts of glycogen present in liver and body at the time of the injections, as calcu-

lated from Table I, are shown in Table II. It will be noted in Table II that saline, epinephrine, and insulin were injected into animals containing very similar quantities of glycogen. The average glycogen content of the liver in the three cases was 220, 218, and 226 mg., and of the rest of the body 432, 427, and 438 mg. The glycogen values observed 3 hours after the injections are recorded in Table I. By subtracting the glycogen values in Table I from those in Table II one obtains the values recorded in Table III.

TABLE III.

Disappearance of Glycogen (in Mg. of Glucose) from Liver and Rest of Body during 3 Postabsorption Hours.

Calculated from Tables I and II.

	Liver glycogen.	Body glycogen.	Loss of sugar from body fluids.	Total carbohydrate disappeared.	Carbohydrate oxidized.
Controls, saline injection.	-44	-133	-15	192	255
	-79	-195	-30	304	169
	-40	-166	-23	229	263
	-33	-175	-18	226	195
Epinephrine injection.	-31	-342	+ 9	364	312
	+34	-313	+17	262	254
	+13	-286	- 5	278	255
	+51	-287	± 0	236	261
	+61	-259	+19	179	231
Insulin injection.	-146	-171	-52	369	400
	-170	-182	-43	395	448
	-141	-231	-45	417	450
	-108	-170	-44	322	437

First a comparison will be made between the carbohydrate oxidation of the glucose absorption and the postabsorptive period. The control animals in Table I oxidized an average of 417 mg. of sugar in the 4 hour glucose absorption period. In the 3 hour postabsorptive period which follows they oxidized 220 mg. of sugar. In order to compare these two values they are calculated per hour; namely, 104 mg. oxidized in the absorption period, and 73 mg. oxidized in the postabsorptive period. This indicates that as soon as the supply of glucose from the intestine subsides, there

is a decrease in carbohydrate oxidation. The same is true for the animals which received epinephrine after the glucose absorption period. In the absorption period they oxidized 104 mg. per hour, against 87 mg. per hour in the postabsorptive period. In contrast to this, the animals which received insulin after the glucose absorption period showed an increase in carbohydrate oxidation. In the absorption period they oxidized 99 mg. per hour; in the postabsorptive period the oxidation rose to 145 mg. per hour.

Secondly, a comparison will be made between the amounts of glycogen disappearing from liver and body during 3 postabsorption hours following saline, epinephrine, and insulin injections. The individual experiments are recorded in Table III. It will be

TABLE IV.

Disappearance of Glycogen (in Mg. of Glucose) from Liver and Rest of Body during 3 Postabsorption Hours.

Average values of Table III.

	Liver glycogen.	Body glycogen.	Loss of sugar from body fluids.	Total carbohydrate disappeared.	Carbohydrate oxidized.	Blood sugar.
Controls, saline injection.....	-49	-167	-22	238	220	113
Epinephrine injection.....	+26	-298	+8	264	263	174
Insulin injection.....	-141	-188	-47	376	434	69

sufficient to consider the average values of Table III which are given in Table IV. The control rats in Table IV utilized 49 mg. of liver and 167 mg. of body glycogen (mainly muscle glycogen). The animals receiving epinephrine utilized apparently no liver glycogen, since there was an increase in liver glycogen of 26 mg. All the glycogen which the animals receiving epinephrine utilized came from the rest of the body. The important point is that nearly twice as much body glycogen disappeared after epinephrine injections than under normal conditions or after insulin injections. The insulinized rats utilized 3 times more liver glycogen but only slightly more body glycogen than the control rats. Contrary to expectation it is not epinephrine but insulin which causes a marked disappearance of liver glycogen in rats in the postabsorptive state.

The insulinized rats utilize the largest amount of liver glycogen, the rats receiving epinephrine the largest amount of body glycogen, while the normal rats hold an intermediate position. The calculated disappearance of carbohydrates shows a fair agreement with the observed oxidation of carbohydrates. In the normal animals in Table IV 7.5 per cent more carbohydrate disappears than is accounted for by oxidation; in the animals receiving epinephrine the agreement happens to be perfect, which is undoubtedly a coincidence, while in the insulinized animals 13.4 per cent more sugar is oxidized than is accounted for by the calculated disappearance of sugar. If insulin would cause the conversion of part of the disappearing sugar into an unknown intermediary substance, more sugar should be lost sight of than is accounted for by oxidation. Here the opposite condition obtains, definitely disproving this hypothesis.

Corresponding to the fall in blood sugar, glucose disappeared from the blood and body fluids of the control and insulinized rats, while glucose accumulated in the rats receiving epinephrine because hyperglycemia developed (Table IV). At the end of the 3 hour postabsorptive period the average blood sugar of the normal rats was 113, of the rats receiving epinephrine 174, and of the insulinized rats 69 mg. per cent (Table I). The dose of epinephrine administered produced no glycosuria. In Rat 5, marked with a dagger in Table I, the double dose of epinephrine (0.04 mg. per 100 gm. of rat) was given, with the result that 10 mg. of sugar were excreted in the urine. It was not intended to produce glycosuria in these experiments; the aim was to keep the dose as small as possible and yet to obtain a clear effect on carbohydrate metabolism. In this way it was found that a dose that would be pronounced small, when judged by its effect on blood sugar, has a very marked effect on the disappearance of muscle glycogen. It is therefore not necessary to inject a large dose in order to demonstrate an extrahepatic action of epinephrine. How far the dose of epinephrine here employed may be regarded as physiological is discussed in the following paper (23).

Influence of Epinephrine and Insulin on Heat Production.

The data on respiratory metabolism in Table I are expressed in terms of calories in Table V. First a comparison will be made

between the average calorie production of the glucose absorption and the postabsorptive period. The control and insulinized animals showed a slightly lower heat production in the postabsorptive period as compared with the absorption period. In the former animals the total calories diminished by 3.6, and in the latter animals by 3.8 per cent. In contrast to this, the heat production rose when epinephrine was injected; namely, from 0.76 calories per hour in the absorption period to 0.86 calories per hour in the postabsorptive period, corresponding to an increase of 12.2 per cent. Next the average calorie production of the normal rats in the postabsorptive state is compared with that of the rats in the

TABLE V.

Influence of Epinephrine and Insulin on Heat Production of Rats in the Postabsorptive State.

Values per 100 gm. of body weight. Calculated from Table I.

	Glucose absorption period (4 hrs.).					Postabsorptive period (3 hrs.).				
	Calories from:			Total calories.	Total calories per hr.	Calories from:			Total calories.	Total calories per hr.
	Protein.	Fat.	Carbohydrate.			Protein.	Fat.	Carbohydrate.		
Control.....	0.29	1.02	1.56	2.87	0.72	0.21	1.03	0.83	2.07	0.69
Epinephrine*.....	0.31	1.19	1.56	3.06	0.76	0.24	1.35	0.98	2.57	0.86
Insulin*.....	0.29	1.09	1.48	2.86	0.71	0.22	0.22	1.62	2.06	0.69

*Injected at the beginning of the postabsorptive period.

postabsorptive state receiving insulin and epinephrine injections. The calories of the control and insulinized animals were within 0.5 per cent the same, in spite of the fact that the latter animals oxidized 97.3 per cent more carbohydrates than the former. The insulinized animals produced 0.79 more carbohydrate and 0.81 less fat calories than the control animals, in other words, there occurred an equicaloric replacement of carbohydrate oxidation for fat oxidation after the insulin injection. This perfect replacement is quite remarkable and it explains why insulin does not have

a calorogenic action.² On the other hand, when epinephrine was injected, the heat production was 24 per cent higher than in the normal animals in the postabsorptive state.³ The animals receiving epinephrine produced 0.32 more fat and 0.15 more carbohydrate calories than the control animals. Therefore, 67 per cent of the extra calories was furnished by fat and 33 per cent by carbohydrates. Since in the control animals only 55 per cent of the non-protein calories was derived from fat, epinephrine increased fat oxidation proportionately more than carbohydrate oxidation. If no carbohydrates are being oxidized at the time of the epinephrine injections, as in the experiments on 24 hour fasting rats (1), the extra heat is furnished exclusively by fat oxidation. The mechanism by which epinephrine increases fat oxidation is still uncertain. There may be a direct accelerating influence on fat oxidation, or fat metabolism may be influenced indirectly through an as yet unknown mechanism.

Utilization of Liver and Body Glycogen in Normal Rats in the Postabsorptive State.

The utilization of liver and body glycogen in normal rats in the postabsorptive state is of interest from the standpoint of the physiology of fasting. The control rats in Table IV utilized 216 mg. of glycogen, 23 per cent being derived from the liver and 77 per cent from the rest of the body. Body glycogen (chiefly muscle glycogen) is therefore utilized to a larger extent in the first 3 postabsorption hours than liver glycogen. When the fast has lasted for 24 hours, neither liver nor body glycogen disappears. The glycogen content of the rats remains practically constant, there being a diminution of only 22 mg. of glycogen between the 24th and 48th hour of fasting (1). It is of interest to inquire how soon this stationary condition of the glycogen is reached and what metabolic adjustments have to take place until the glycogen can remain constant. If the utilization of liver and body glycogen were going on at the same rate in later periods of fasting as in the first 3 post-

² Hawley and Murlin (24) observed in rabbits increased heat production from fat in the 1st hour of insulin action but not in later periods. This would escape attention in our experiments which extend over 3 hours.

³ Boothby and Sandiford (25) using dogs, observed an increased heat production of a similar magnitude after physiological doses of epinephrine.

absorption hours, the 24 hour fasting level would be reached by the liver in 13 and by the body in 5 hours. There is reason to believe that the rate of glycogen utilization diminishes as fasting proceeds, because the insulin production of the pancreas will adjust itself to the fasting condition. Utilization of liver glycogen is greatly increased when insulin is present in excess during fasting. It will be noted in Table IV that the insulinized rats utilized 3 times more liver glycogen than the normal rats in the postabsorptive state. A decrease in the insulin production of the pancreas which is known to occur during fasting⁴ will therefore make for a longer preservation of liver glycogen. At or before 24 hours of fasting most of the liver glycogen is used up and the body glycogen has attained a stationary condition. If the body glycogen is to reach this stationary state, carbohydrate oxidation has to be replaced by fat oxidation. Presumably, this metabolic replacement cannot be carried to completion until the insulin production of the pancreas has been reduced to a minimum. Since insulin and epinephrine act antagonistically, a low supply of the former may mean a relative preponderance of the latter. It is possible that the preservation of a normal blood sugar level during fasting is in some way connected with the epinephrine discharge of the adrenals. This is suggested by the observation that adrenalectomized rats developed hypoglycemia after a fasting period of 24 hours and that the livers of such animals contained only traces of glycogen (26). A similar observation was made on adrenalectomized mice after shorter periods of fasting and in three cases typical hypoglycemic convulsions developed after 5 hours of fasting. The cycle of carbohydrates which is observed after small doses of epinephrine (see the following discussion) may play a rôle in the preservation of liver glycogen and hence of a normal blood sugar level during fasting. This cycle proceeds in the following manner. Lactic acid derived from muscle glycogen is converted into liver glycogen and glucose derived from liver glycogen is returned to the muscles. In the absence of carbohydrate oxidation this cycle can go on without any loss of carbohydrate and may therefore explain why glycogen is always found in the liver of fasting animals, making unnecessary the assumption that gluconeogenesis occurs.

⁴Staub, H., *Z. klin. Med.*, 1926, civ, 587.

Influence of Insulin and Epinephrine on Utilization of Liver and Body Glycogen.

The mere determination of the respiratory metabolism after insulin or epinephrine injections does not permit of a conclusive analysis of the changes in carbohydrate metabolism produced by these hormones. In the experiments in Table IV insulin increased carbohydrate oxidation by 97.3 and epinephrine by 19.5 per cent. The fact that insulin and epinephrine have an accelerating effect on carbohydrate oxidation but an opposite effect on the blood sugar level would be difficult to explain without knowledge of the underlying changes in liver and body glycogen. The increased carbohydrate oxidation after non-convulsive doses of insulin does not occur to any appreciable extent at the expense of muscle glycogen but almost exclusively at the expense of liver glycogen. It will be noted in Table IV that the animals receiving insulin oxidized only 21 mg. more of body glycogen than the normal rats in the postabsorptive state, while 3 times more liver glycogen disappeared. The rapid disappearance of liver glycogen after insulin injections is regarded as an indirect effect. It is brought about by an increased utilization of blood sugar in the peripheral tissues with a compensatory mobilization of liver glycogen. This action of insulin on rats in the postabsorptive state is analogous to the one observed on sugar-fed rats (22), where the muscles appropriated most of the absorbed sugar at the expense of glycogen deposition in the liver. According to this analysis, insulin is a hormone that leads to a preferential utilization of blood sugar and indirectly of liver glycogen, the latter being the only important direct source of blood sugar in the body.

After epinephrine injections, carbohydrate oxidation is carried on mainly at the expense of muscle glycogen. In the experiments on rats in the postabsorptive state in Table IV, the disappearance of muscle glycogen was greatly accelerated, while liver glycogen was apparently not drawn upon since it did not diminish. Utilization of blood sugar (which is derived from liver glycogen) and of muscle glycogen are therefore influenced in an opposite direction by epinephrine injections. The former is diminished, while the latter is markedly increased in rats in the postabsorptive state. This explains the otherwise unintelligible fact that epinephrine

may cause hyperglycemia and increased carbohydrate oxidation at the same time.

An increase in carbohydrate oxidation after epinephrine is observed only in rats in the postabsorptive state but not in sugar-fed or in fasting rats. The influence of epinephrine on sugar-fed animals will be discussed in a later paper (23). In 24 hour fasting rats in which carbohydrate oxidation is reduced to a minimum or absent, epinephrine injections do not lead to carbohydrate oxidation though they cause a disappearance of muscle glycogen. This indicates, together with the results obtained on sugar-fed animals, that epinephrine has not a direct accelerating influence on carbohydrate oxidation. It merely mobilizes muscle glycogen, and it depends on the metabolic state of the animal (presumably on the insulin content of the tissues) to what extent the glycogen mobilized in the muscles is being oxidized.

Carbohydrate Balance after Epinephrine.

The glycogen content of the liver observed 3 hours after the epinephrine injection may be represented as the algebraic sum of the following processes.

1. Mobilization of liver glycogen.
2. Reconversion of blood sugar into liver glycogen.
3. Conversion of blood lactic acid (from muscle glycogen) into liver glycogen.

Since glycogen disappears from the liver of the normal rat in the postabsorptive state and since there is no indication that epinephrine inhibits glycogenolysis in the liver, Process 1 must occur though the sugar balance in Table IV offers no evidence thereof. Epinephrine does not prevent Process 2, since it has no inhibiting effect on glycogen synthesis in the liver during glucose absorption (23). Process 3 has already been discussed in the preceding paper (1). Its occurrence in the rat in the postabsorptive state following epinephrine injections is indicated by the fact that more glycogen disappears from the muscles than can be accounted for by carbohydrate oxidation (Table IV). Under three different experimental conditions (24 hour fasting, rats in the postabsorptive state, and sugar-fed rats) the algebraic sum of these three processes was positive, that is, epinephrine caused an increase and not a decrease in liver glycogen. Here only the rats in the postabsorptive state

will be considered. We can leave Process 2 out of consideration for the present, because it is a function of Process 1, there being no other direct source of blood sugar than liver glycogen. The question arises as to what rate should be assigned to Processes 1 and 3. The sugar balance merely gives information as to the minimum rate of Processes 1 and 3. This is shown in the following calculation. In the experiments in Table IV the liver glycogen increased by 26 mg. At the same time 298 mg. of glycogen disappeared from the body, while only 263 mg. of sugar were oxidized. This leaves 35 mg. of glycogen for Process 3. In order to raise the blood sugar to 174 mg. per cent, a mobilization of 8 mg. of liver glycogen is required. Deducting 8 mg. from 35 mg. one obtains the end result of plus 27 mg. of liver glycogen, 26 mg. being the figure actually observed. The conditions of the sugar balance are fulfilled but no room is left for mobilization of liver glycogen and hence for utilization of blood sugar in the tissues. If one allows for blood sugar utilization, more liver glycogen must be mobilized to supply sugar, and consequently, since the liver glycogen is not found to diminish, a greater rate of Process 3 is required. In this way a cycle of carbohydrates is established. Blood sugar derived from liver glycogen is utilized in the muscles and at the same time lactic acid derived from muscle glycogen is transported back to the liver to replenish the glycogen which has been lost.⁵

It is evident that the rate of this cycle is determined by the rate of blood sugar utilization. In the normal and insulinized animals, in which Process 3 is not occurring, the amount of liver glycogen that disappears can be used as an index of blood sugar utilization. There was a disappearance of 49 mg. in the control and of 141 mg. in the insulinized animals (Table IV). To these must be added 22 mg. of sugar derived from the body fluids in the former and 47 mg. in the latter animals, giving a total blood sugar utilization of 71 and 188 mg. respectively. As has already been pointed out, epinephrine certainly does not inhibit glycogen mobilization in the liver. If therefore as much liver glycogen is mobilized as in

⁵ This cycle is a necessary supposition, if utilization of blood sugar occurs after epinephrine injections, unless one makes the assumption that liver glycogen is formed from non-carbohydrate sources as fast as it disappears. As has been set forth in the preceding paper (1) there is no convincing evidence for the second alternative.

the normal rats in the postabsorptive state, 49 mg. of sugar may be utilized in the peripheral tissues of the rats receiving epinephrine injections. This requires a return of 73 mg. of lactic acid from the muscles in order to account for the end result of plus 26 mg. of liver glycogen. The mobilization of liver glycogen will be greater than 49 mg. in case blood sugar is reconverted into liver glycogen (Process 2). Process 2 can only occur to any larger extent in animals in the postabsorptive state following epinephrine injections, if part of the sugar mobilized in the liver fails to be utilized in the peripheral tissues. That blood sugar utilization is low during epinephrine hyperglycemia is suggested by the results obtained in some recent experiments on men (unpublished). Under postabsorptive conditions venous blood of a limb contains 4 to 8 mg. less sugar than arterial blood. When sugar is ingested (Foster (27)), or insulin injected (28), or most markedly, when sugar plus insulin is administered, the arteriovenous difference rises to 30 mg. and more, indicating that the peripheral tissues take up more blood sugar under these conditions. However, when hyperglycemia is produced by epinephrine injections, the arteriovenous difference remains unchanged, indicating that the peripheral tissues are unable to withdraw blood sugar at a higher rate.

DISCUSSION.

According to the generally accepted explanation epinephrine causes hyperglycemia, and after large doses, glycosuria, merely because it mobilizes liver glycogen. As pointed out in the introduction, hyperglycemia and glycosuria cannot be explained on this basis because there is not enough liver glycogen present to provide the tissues for hours with sugar at a rate which is greater than their normal ability to utilize sugar. This is illustrated by the following calculation. In the experiments in Table IV the average blood sugar was 174 mg. per cent 3 hours after the epinephrine injection. In order to produce a hyperglycemia of this magnitude in normal rats, glucose must be infused intravenously at a rate of 250 mg. per 100 gm. of body weight per hour (29). Hence, a mobilization of 750 mg. of liver glycogen or an initial glycogen content of the liver of 20 per cent is required, if the hyperglycemia of 174 mg. per cent is to be explained merely on the basis of glycogen mobilization in the liver. Actually there were only 218

mg. or 5.6 per cent liver glycogen present to start with (Table II), and the liver glycogen showed an increase after the epinephrine injection. This indicates that other factors play a rôle in the production of hyperglycemia besides mobilization of liver glycogen. In fact the experiments with epinephrine in Table IV, when taken at their face value, give no evidence that liver glycogen is being mobilized; only by means of a finer analysis could it be shown that mobilization of liver glycogen occurs.

Decreased utilization of blood sugar is one of the principal causes for the epinephrine hyperglycemia under postabsorptive conditions. The blood sugar rises after epinephrine injections, because the utilization of blood sugar in the peripheral tissues falls behind the supply of blood sugar by the liver, even if the supply is not much higher than normal. For this reason, amounts of sugar given off by the liver will cause hyperglycemia, amounts, which when injected into normal animals, have no effect whatever on the blood sugar level. Hitherto it has been assumed that a decreased utilization of blood sugar must be associated with a decreased carbohydrate oxidation. The present experiments reveal that after epinephrine injections carbohydrate oxidation is carried on at the expense of muscle glycogen and that the carbohydrate oxidation may be increased in spite of a diminished blood sugar utilization.⁶

The conversion of lactic acid derived from muscle glycogen into liver glycogen explains why epinephrine hyperglycemia can persist for several hours without causing a depletion of the glycogen stores of the liver. A decrease in liver glycogen is to be expected only if glycosuria develops. In rabbits during epinephrine glycosuria more sugar appears in the urine than can be accounted for by the disappearance of liver glycogen, indicating that resynthesis of liver glycogen from lactic acid occurs also in this species (30). It can now be understood why different observers obtained conflicting results when they determined the glycogen content of the liver after epinephrine injections. With small doses of epinephrine, or even with large doses when the glycogen content of the liver is low, resynthesis of liver glycogen from lactic acid exceeds mobilization, and consequently the liver glycogen is found higher after the injection. With large doses and a high liver gly-

⁶ It is therefore not correct to speak of "adrenalin diabetes."

cogen the latter diminishes because the loss of sugar in the urine cannot entirely be compensated by resynthesis of liver glycogen.

It is surprising that an extrahepatic action of epinephrine on carbohydrate metabolism has not been emphasized before. The experiments on 24 hour fasting and on rats in the postabsorptive state indicate that the extrahepatic action of epinephrine consists in a mobilization of muscle glycogen. Whether this mobilization is responsible for the decreased utilization of blood sugar, or whether both processes are influenced independently by epinephrine, cannot be decided at present.

Since the end-product of glycogen breakdown in the muscle is lactic acid, epinephrine cannot raise the blood sugar level after removal of the liver. However, the influence of epinephrine on blood sugar utilization is demonstrable on hepatectomized animals, as will be shown in subsequent papers.

Insulin produces hypoglycemia because the supply of sugar falls behind blood sugar utilization, in spite of a rapid mobilization of liver glycogen. Epinephrine and insulin antagonize each other on account of their opposite effect on blood sugar utilization in the peripheral tissues.

SUMMARY.

1. A carbohydrate balance has been made on rats in the post-absorptive state by determining simultaneously sugar oxidation and disappearance of glycogen from the liver and the rest of the body. In the control rats 7.5 per cent more carbohydrate disappeared than was accounted for by oxidation; in the rats injected with epinephrine the agreement happened to be perfect; in the rats injected with insulin 13.4 per cent more carbohydrate was oxidized than could be accounted for by the disappearance of glycogen.

2. In the control rats 216 mg. of glycogen disappeared in 3 hours, 23 per cent being derived from the liver and 77 per cent from the rest of the body. In the animals injected with epinephrine all the glycogen which disappeared (298 mg.) came from the body, there being an actual increase in liver glycogen of 26 mg. After insulin 43 per cent of the disappearing glycogen (329 mg.) was derived from liver and 57 per cent from body glycogen.

3. In comparison with the control rats, the animals receiving

epinephrine showed an increase in carbohydrate oxidation of 19.5 per cent and the animals receiving insulin of 97.3 per cent. The blood sugar level in the three cases stood at 113, 174, and 69 mg. per cent respectively. Glycosuria was not produced by the dose of epinephrine injected (0.02 mg. per 100 gm. of rat).

4. The calories of the control and insulinized animals were within 0.5 per cent the same, indicating that there occurred an equicaloric replacement of carbohydrate oxidation for fat oxidation after the insulin injection. Epinephrine caused an increase in heat production of 24 per cent, 67 per cent of the extra calories being furnished by fat and 33 per cent by carbohydrate.

5. The mechanism of epinephrine action is discussed. After non-glycosuric doses the most prominent effect of epinephrine is observed in the peripheral tissues and consists in a mobilization of muscle glycogen and in a decreased utilization of blood sugar. Oxidation of muscle glycogen is increased under postabsorptive conditions, sharply distinguishing the action of epinephrine from the effects of pancreatectomy. Part of the lactic acid arising from the breakdown of muscle glycogen escapes removal by oxidation and is carried to the liver where it is deposited as glycogen. In this way a cycle of carbohydrates is established. Blood sugar derived from liver glycogen is utilized in the muscles and lactic acid derived from muscle glycogen is returned to the liver. Epinephrine hyperglycemia develops because the utilization of blood sugar is diminished in relation to the supply of blood sugar by the liver, even if the supply is not much higher than normal. Insulin is the hormone that leads to a preferential utilization of blood sugar and indirectly of liver glycogen. The antagonistic action between insulin and epinephrine is explained by their divergent effect on blood sugar utilization.

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THE MECHANISM OF EPINEPHRINE ACTION.

III. THE INFLUENCE OF EPINEPHRINE ON THE UTILIZATION OF ABSORBED GLUCOSE.

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The experiments in the preceding paper (1) were made on rats in the postabsorptive state. Under postabsorptive conditions blood sugar is being supplied by the liver, only a small amount of sugar is transported by the blood, glycogen synthesis is not taking place to any large extent, and carbohydrate metabolism is carried on mainly at the expense of preformed glycogen. These experimental conditions are favorable for observing the influence of epinephrine on glycogen utilization and it has been found that epinephrine injections cause a more rapid disappearance of muscle glycogen and an increase in liver glycogen in rats in the post-absorptive state. The present experiments on sugar-fed rats offer a different problem. During glucose absorption a large amount of sugar passes into the blood. This sugar must penetrate into the tissues prior to its utilization. In the tissues the sugar is disposed of, partly by oxidation, partly by conversion into glycogen, and carbohydrate metabolism is carried on mainly at the expense of the sugar that is circulating in the blood. These experimental conditions are favorable for observations on the influence of epinephrine on blood sugar utilization. The uptake of blood sugar was diminished in rats in the postabsorptive state following epinephrine injections. This effect of epinephrine should be more marked in sugar-fed rats, since during intestinal absorption 14 times more sugar is transported by the blood than under postabsorptive conditions. It was actually found that the same dose of epinephrine that produced only hyperglycemia in rats in the postabsorptive state, caused a marked glycosuria

in sugar-fed rats. As will be shown later this glycosuria is mainly due to a decreased utilization of blood sugar in the peripheral tissues.

Methods.

In the present paper a comparison is made between the utilization of glucose in normal rats and in rats receiving epinephrine injections. The control series, consisting of ten individual experiments, has already been published in Table III of a previous paper (2), where all the necessary details are given. Both the control and the experiments with epinephrine were made within 2 months (March and April, 1927) and care was taken to spread them evenly over this period. In order to save space the control series is not reproduced again, with the exception of the average values which are given in Table III of the present paper. The technique used has also been described in the previous paper (2). In brief outline the experiments were performed in the following way. Male rats of 140 to 150 gm. of body weight were fasted for 22 hours. A metabolism fore period of 2 hours was made in a Haldane type metabolism apparatus. Enough glucose was then fed by stomach tube to permit the absorption to proceed for at least 5 hours, and the subcutaneous injection of epinephrine was made (0.02 mg. per 100 gm. of body weight). The respiratory metabolism was determined for 4 hours after the sugar feeding. The animals were then killed, and absorption and glycogen content of the liver and the rest of the body were determined in the usual manner. The glycogen values found after the 4 hours of glucose absorption were corrected for the preformed glycogen, 140 mg. being subtracted for the body and 7 mg. for the liver (see Table I of a previous paper (2)). The second metabolism period was made in a closed type of metabolism apparatus, which was used for numerous experiments on previous occasions, and which permits a determination of O_2 consumption by means of two independent methods. The average difference between the two O_2 determinations for the eight metabolism experiments here reported was 1.4 ± 0.8 per cent. Calculations of the amounts of sugar and fat oxidized were made from the non-protein R.Q. with the aid of the Zuntz-Schumburg-Lusk tables. The control rats were treated in exactly the same way except that no epinephrine was given.

Epinephrine injections have been shown to cause overventilation (3), which in turn may lead to the blowing off of CO_2 and consequently to R.Q.'s which are too high. After the period of overventilation the R.Q.'s may be found too low, owing to a retention of CO_2 . The danger of erroneous R.Q.'s on account of disturbances in respiration is great in metabolism experiments of short duration. However, this danger is not met with in metabolism experiments extending over 4 hours, partly because the amount of CO_2 that could be blown off would be small in comparison to the amount of CO_2 arising from the metabolism during that time, partly because enough time elapses in which a disturbance of the acid-base equilibrium might be readjusted. Previous experiments (4) have shown that large doses of epinephrine cause an increase in the lactic acid content of the blood of rabbits and cats. In view of this, CO_2 determinations were made on the blood of control rats under the conditions obtaining at the beginning and end of the metabolism experiments with epinephrine. 4 hours after glucose feeding plus epinephrine, CO_2 values (for plasma saturated with alveolar air) of 51.4 to 56.7 volumes per cent were obtained. This in no way departs from the values found before epinephrine was injected. The acid-base balance was, therefore, the same at the beginning and end of the metabolism experiments with epinephrine, which is of importance for the interpretation of the R.Q.'s. Whether there was any change in plasma CO_2 at shorter time intervals after the injection was not ascertained.

Epinephrine Dose.

The dose used in the present work was the same as in the experiments on 24 hour fasting and rats in the postabsorptive state; namely, 0.02 mg. per 100 gm. of body weight, injected subcutaneously in 0.1 cc. of fluid. The dilution was prepared from the 1:1000 adrenalin chloride solution of Parke, Davis and Company. It is of importance to decide whether this dose is small or large in a physiological sense. The following points must be taken into consideration. There is good evidence that subcutaneously injected epinephrine is not absorbed at once into the blood stream. Epinephrine blocks its own passage into the circulation because of local vasoconstriction, and absorption can probably proceed only at the border of the anemic area. In this way a depot is

created in the subcutaneous tissue. Absorption from this depot can persist for several hours, because epinephrine is not rapidly destroyed in the subcutaneous tissue (Auer and Meltzer (5), Tatum (6), Luckhardt and Koppányi (7)). After the subcutaneous injection of 1 mg. of epinephrine per kilo, as a rule, no rise in blood pressure is observed in rabbits, cats, and dogs. Trendelenburg (8) concluded from this that the rate of absorption after such an injection must be below 0.0005 mg. of epinephrine per kilo per minute, because a constant intravenous infusion at this rate is just able to cause a rise in blood pressure of unnarcotized rabbits. Cannon and Rapport (9) found an average maximal rate of discharge of 0.003 mg. of epinephrine per kilo per minute from the adrenals of the cat. It appears that the rate at which subcutaneously injected epinephrine is absorbed into the blood stream is lower than the maximal rate of discharge of epinephrine from the adrenals. Trendelenburg (10) found in unanesthetized rabbits that glycosuria is produced at a rate of infusion of epinephrine which has no effect on blood pressure. Straub (11) and Tatum (6) have shown that glycosuria and hyperglycemia persist only as long as epinephrine enters the blood stream. This is mainly due to the fact that epinephrine is almost entirely destroyed upon one passage through the capillaries. Since in the present experiments the glycosuria of the rats persisted for 4 hours, epinephrine must have been absorbed for at least that length of time. The average sugar excretion from 0 to 1, from 1 to 2, from 2 to 3, and from 3 to 4 hours was 12, 13, 15, and 15 mg. respectively (Tables I and III). The investigation was not carried beyond 4 hours. The fact that the amounts of sugar excreted from hour to hour remained practically constant, suggests a rather constant rate of absorption of epinephrine from the subcutaneous tissue. Assuming that all the epinephrine injected, namely 0.02 mg. per 100 gm. of rat, was absorbed during the 4 hours, which it probably was not, one obtains a rate of absorption of $\frac{0.2}{240} = 0.0008$ per kilo per minute. This is lower than the average maximal output of the adrenals of the cat as determined by Cannon and Rapport. The rate of discharge of epinephrine from the adrenals of the rat is not known. The following figures are of interest in this connection. According to Herring (12) and Kuriyama (13) there are

0.034 to 0.043 mg. of epinephrine in the adrenals of a rat of 100 gm. of body weight, while in the present experiments at the most 0.02 mg. of epinephrine passed into the blood in the course of 4 hours. These points were discussed at some length in order to show that the rate at which epinephrine is absorbed from the subcutaneous tissue of the rat, when 0.02 mg. in 0.1 cc. of fluid is injected, falls within physiological limits. For this reason the results obtained with this dose might be expected to be of physiological significance.

Changes in Carbohydrate Metabolism 1, 2, and 3 Hours after Injection.

A carbohydrate balance was made for the 4 hour absorption period. It was of interest to investigate whether certain characteristic changes which are present 4 hours after the injection, can also be observed at earlier periods. Table I contains data on absorption, glycogen content of liver, and blood and urinary sugar of control and injected rats 1, 2, and 3 hours after glucose feeding. The respective values for the 4 hour absorption period may be found in Table II. It should be noted that the average weight of the rats in Table I was greater than that of the rats used for the 4 hour period. The highest average blood sugar (299 mg. per cent) was observed 1 hour after the injection. After 2 and 3 hours the blood sugar was lower (251 and 257 mg.), and a further fall in blood sugar occurred between the 3rd and 4th hour. In spite of this decrease in blood sugar the rate of sugar excretion in the urine remained practically constant from hour to hour.

The rats receiving epinephrine absorbed less glucose than the controls. This is noticeable after 2, 3, and 4 hours but not after 1 hour of absorption. An explanation for the slower rate of absorption cannot be given at present. It remains undecided whether in these experiments epinephrine had any inhibiting effect on intestinal motility, and furthermore, it is not known whether a decreased motility reduces the rate of absorption. Constriction of splanchnic blood vessels might also be considered. Neither of these explanations fits the fact that only 1 hour after the injection did the inhibition of absorption reach a marked degree. These observations suggest that an increased release of epinephrine from the adrenals may influence absorption.

Though the rats receiving epinephrine absorbed less glucose they

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formed more liver glycogen than the control rats. With the exception of the 1st hour, the difference in glycogen deposition is significant as shown by the average deviation from the mean (Table I). The sugar balance for the 4 hour absorption period shows that the increased deposition of liver glycogen is not due to

TABLE I.
Influence of Epinephrine on Glucose Absorption, Glycogen Formation in Liver, and Sugar Excretion.

Average body weight 165.5 ± 10 gm. Values calculated per 100 gm. of body weight.

Length of absorption period.	Controls.			Epinephrine.			
	Glucose absorbed.	Glycogen formed in liver.	Blood sugar.	Glucose absorbed.	Glycogen formed in liver.	Blood sugar.	Urine sugar.
hrs.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
1	193	26		225	33	324	8
	259	28	137	182	43	267	13
	204	41	131	181	31	298	8
	213	36	126	229	27	307	18
2	217 ± 21	33 ± 6	131	204 ± 23	34 ± 5	299	12
	452	68	153	344	73	217	21
	318	55	159	318	97	321	21
	420	82	156	254	79	226	31
	370	68	144	270	81	241	27
3	390 ± 46	68 ± 7	153	296 ± 33	83 ± 7	251	25
	570	141	156	495	147	243	39
	564	156	161	543	185	251	25
	666	118	148	507	151	254	49
	627	101	149	498	172	261	67
	624	112	142	486	171	276	21
	609 ± 35	126 ± 18	151	507 ± 16	165 ± 13	257	40

an accelerating effect of epinephrine on the synthetic process. The actual mechanism involved will be discussed in the next section.

Sugar Balance after Epinephrine.

The individual experiments of the 4 hour period in Table II show that the results are uniform throughout. It will be sufficient

TABLE II.
Influence of Epinephrine on Sugar Oxidation and Glycogen Formation during 4 Hours of Glucose Absorption.
 Average body weight 134.7 ± 3 gm. Values calculated per 100 gm. of body weight per 4 hours.

Fore period.		Absorption period.											
O ₂	x.q.	Glucose absorbed.	Sugar oxidized.	Glycogen formed.			Glucose recovered.		O ₂	x.q.	Urine N.	Urine sugar.	Blood sugar.
				In liver.	In rest of body.	Total.	gm.	per cent					
gm.		gm.	gm.	gm.	gm.	gm.	gm.		gm.		mg.	gm.	mg.
0.897	0.709	0.795	0.270	0.164	0.109	0.273	0.628	79.0	1.058	0.794	10.11	0.085	189
0.931	0.723	0.870	0.323	0.256	0.090	0.346	0.716	82.3	0.966	0.823	14.55	0.047	202
0.941	0.705	0.953	0.330	0.255	0.153	0.408	0.761	79.8	1.084	0.812	14.69	0.023	215
1.108	0.711	0.897	0.351	0.184	0.148	0.332	0.745	83.1	1.115	0.816	14.59	0.062	189
0.898	0.709	0.957	0.360	0.181	0.196	0.377	0.768	80.3	0.985	0.833	15.36	0.031	188
0.974	0.703	0.891	0.361	0.267	0.091	0.358	0.784	88.0	1.036	0.823	10.85	0.065	232
0.883	0.719	0.953	0.387	0.213	0.179	0.392	0.832	87.3	0.999	0.840	16.13	0.053	190
1.017	0.709	0.973	0.474	0.173	0.119	0.292	0.843	86.6	1.099	0.852	14.68	0.077	176

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to consider the average values in Table III. The recovery of absorbed sugar amounts to 83.4 per cent in the experiments with epinephrine and to 86.5 per cent in the control experiments. The lower recovery in the former case is due to the fact that the blood

TABLE III.
Average Values Calculated from Table II.

The values for the controls are taken from a previous paper (2).

	Glucose.	Glucose plus epinephrine.
Fore period.		
O ₂ , gm.....	0.954	0.956
R. Q.....	0.715	0.712
Total calories.....	3.09	3.10
Glucose absorption period.		
Glucose absorbed, gm.....	1.065	0.911
" oxidized "	0.465	0.357
Glycogen formed in liver, gm.....	0.192	0.212
" " " rest of body, gm.....	0.263	0.136
Total glycogen, gm.....	0.455	0.348
Glucose recovered, gm.....	0.919	0.760
" " per cent.....	86.5	83.4
" lost, gm.....	0.146	0.159
Glycogen formed	1.00	0.97
Sugar oxidized		
Blood sugar, mg.....	158	198
Urine " gm.....		0.055
" N, mg.....	15.65	13.87
O ₂ , gm.....	0.885	1.043
R.Q.....	0.884	0.824
Non-protein O ₂ , gm.....	0.752	0.838
" R.Q.....	0.900	0.828
Protein oxidized, gm.....	0.096	0.085
Fat oxidized, gm.....	0.090	0.190
Calories from protein	0.39	0.35
" " fat.....	0.85	1.79
" " sugar.....	1.74	1.33
Total calories.....	2.98	3.47

sugar is higher at the close of the experiment. Consequently more sugar is retained in the body fluids. Assuming that the blood sugar is in equilibrium with 50 per cent of the body weight, one accounts for an additional 20 mg. of sugar. The recovery in the experi-

ments with epinephrine is then 85.6 per cent which is close to that of the control experiments.

Owing to the difference in absorption of the control and injected rats, their sugar oxidation and glycogen formation cannot be compared directly. The respective values have to be calculated per 100 parts of absorbed sugar which eliminates the differences in absorption. This has been done in Table IV, with the data of Table III.

It will be noted in Table IV that the injected rats excreted 6 per cent of the absorbed glucose, while the control rats showed no glycosuria. The same dose of epinephrine, when injected into animals in the postabsorptive state, produced only hyperglycemia

TABLE IV.
Percentage of Sugar Oxidation and Glycogen Formation per 100 Parts of Absorbed Sugar.

Calculated from Table III.

	Parts per 100 parts of absorbed sugar.				
	Oxidized.	Deposited as liver glycogen.	Deposited as glycogen in rest of body.	Retained in body fluids.	Excreted in urine.
Controls.....	44	18	25	2.0	0
Epinephrine.....	39	23	15	5.0	6
Difference.....	-5	+5	-10	+3.0	+6

but no glycosuria (1). Yet, the rats in the postabsorptive state contained a large amount of liver glycogen (5.6 per cent) at the time of the injection while the rats in the present experiments contained practically no liver glycogen to start with. Nevertheless the sugar-fed rats excreted sugar in the 1st hour after the injection. The glycosuria of the sugar-fed rats is therefore related to the transportation of large amounts of sugar by the blood stream. In order to make clear how much sugar is transported in relation to the blood volume, the following example is offered. A rat of 100 gm. of body weight has a blood volume of approximately 7 cc., containing 7 mg. of sugar at a blood sugar level of 100 mg. per cent. In 4 hours 1065 mg. of glucose, or 152 times more sugar than is held by the blood, pass into it from the intestine. The

disposal of sugar in the tissues must therefore be very rapid and must be well adjusted to the rate of absorption in order to keep the blood sugar near the normal level. This is realized in the normal rat, where the blood sugar rises from 100 to 158 mg. per cent during an absorption period of 4 hours, corresponding to a retention of only 29 mg. of sugar in the body fluids out of the 1065 mg. of sugar which have passed into the blood. The rats receiving epinephrine, though they absorbed less sugar than the normal rats, showed a marked rise in blood sugar and in consequence of this, glycosuria. Obviously epinephrine must have interfered with the processes of sugar disposal in the tissues. Since the liver glycogen was higher after the epinephrine injection, the disturbance in sugar utilization must be sought for in the peripheral tissues.

Table IV indicates that the injected rats formed 10 parts less body glycogen than the control rats. It has not been ascertained whether this is due to an actual inhibition of glycogen synthesis in the muscles or whether part of the glycogen already deposited is mobilized again by epinephrine. The second alternative would be consistent with the mobilization of muscle glycogen which has been observed in 24 hour fasting rats and rats in the postabsorptive state after epinephrine injections. To the 10 parts of absorbed sugar which failed to be deposited as body glycogen must be added the 5 parts of absorbed sugar which are not oxidized (Table IV). This leaves a total of 15 parts of absorbed sugar which fail to be utilized in the peripheral tissues and which are therefore accumulating in the blood. 5 parts out of the 15 parts are stored in excess in the liver, 6 parts are excreted in the urine, and 3 parts are retained in the blood and body fluids, leaving 1 part unaccounted for. It is obvious that if the liver were able to store 15 parts in excess instead of only 5 parts, hyperglycemia and glycosuria would not develop. Either the liver is unable to store so large an excess of sugar or part of the glycogen deposited in excess is mobilized again by epinephrine. In both cases the liver plays only a secondary rôle in the production of glycosuria. The factor of primary importance is the excess of sugar accumulating in the blood on account of a decreased utilization of blood sugar in the peripheral tissues. The second factor is the inability of the liver to handle this excess of sugar, possibly because mobilization of

liver glycogen is taking place. This analysis shows that the glycosuria of the sugar-fed rats is mainly the result of an extra-hepatic and not of a hepatic action of epinephrine. In this way the surprising observation that glycosuria develops during a period of increased glycogen storage in the liver finds a satisfactory explanation.

A factor which undoubtedly enters into the present experiments is an increased release of insulin from the pancreas, called forth by the large amounts of sugar passing through the blood. This insulin production enables the normal animal to utilize glucose at a rate of 2.5 gm. per kilo per hour for 4 hours without excreting sugar in the urine. It cannot be expected that under these conditions the small dose of epinephrine injected will entirely suppress the insulin action. The influence of larger doses of epinephrine has not been tried but it seems doubtful whether any dose will have this effect. It is of interest that during glucose absorption epinephrine injections have on every factor entering into the sugar balance an effect opposite to that of insulin injections. With insulin glycogen formation in the liver is decreased, with epinephrine increased. Oxidation and glycogen formation in the peripheral tissues are increased after insulin, decreased after epinephrine. Whether or not these two hormones act antagonistically by influencing the same process in an opposite direction must remain undecided as long as the actual point of attack on the carbohydrate molecule is unknown.

Influence of Epinephrine on Heat Production.

The following authors investigated the effect of epinephrine during sugar absorption. Wilenko (14) observed no rise in R.Q. in rabbits under urethane anesthesia after oral or subcutaneous administration of glucose plus epinephrine. He concluded that epinephrine decreases the ability of the body to oxidize sugar. This has been contradicted by Lusk and Riche (15), since they observed R.Q.'s of 0.9 in dogs after the ingestion of 50 gm. of glucose with simultaneous injection of large doses of epinephrine. The dogs were very restless and the authors therefore did not feel justified in drawing any conclusions as to the calorogenic action of epinephrine. In the present experiments with epinephrine the average R.Q. of the 4 hour glucose absorption period was 0.824 as

compared with the R.Q. of the fore period of 0.712. An increased heat production was observed in each experiment (Table II). The calories rose on an average from 3.1 in the fore period to 3.47 in the glucose absorption-epinephrine period, corresponding to an increase of 12 per cent (Table III). The control rats produced 3.09 calories in the fore period and 2.98 calories in the glucose absorption period, corresponding to a decrease of 3.5 per cent. During glucose absorption the injected rats produced 16.4 per cent more calories than the uninjected controls. In order to ascertain what foodstuff furnished the extra calories after the epinephrine injection, one has to compare the heat production of the glucose absorption period of the control rats with that of the injected rats. The control rats produced 0.85 fat and 1.74 carbohydrate calories, while the injected rats produced 1.79 fat and 1.33 carbohydrate calories. It is therefore evident that the increased heat production after epinephrine is entirely at the expense of fat oxidation. It seems significant that epinephrine is able to accelerate fat oxidation during a period of carbohydrate plethora, though the data so far available do not warrant the conclusion that there exists a direct relationship between the medullary hormone and fat metabolism.

SUMMARY.

1. Glucose was fed to 24 hour fasting rats and 0.02 mg. of epinephrine per 100 gm. of body weight was injected subcutaneously in 0.1 cc. of fluid. An estimate was made of the rate of absorption of epinephrine from the subcutaneous tissue after the injection of such a dose. Hyperglycemia and glycosuria were present after 1 hour and persisted for 4 hours after the injection. The sugar excretion in the urine remained practically constant from hour to hour. Absorption of glucose from the intestine was diminished. With the exception of the 1st hour, where there was no difference, the rats injected with epinephrine deposited more liver glycogen than the control rats.

2. A carbohydrate balance was made for the 4 hour absorption period. Per 100 parts of absorbed sugar, the rats injected with epinephrine deposited 10 parts less body glycogen and oxidized 5 parts less sugar than the control rats. This leaves 15 parts of absorbed sugar which failed to be utilized in the peripheral tissues. 5 parts out of the 15 parts were stored in excess in the liver, 6

parts were excreted in the urine, and 3 parts were retained in the body fluids. The observation that glycosuria develops during a period of increased glycogen deposition in the liver finds its explanation in a decreased utilization of blood sugar in the peripheral tissues.

3. During glucose absorption the rats injected with epinephrine produced 16.4 per cent more calories than the control rats, the extra heat being furnished exclusively by fat oxidation.

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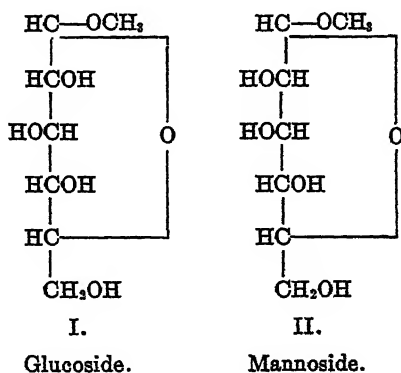
MONOACETONE γ -METHYLGLUCOSIDE.

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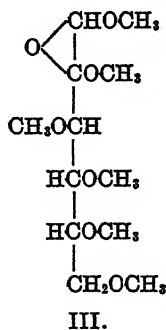
Some time ago¹ we made the observation that α -methyl $<1,5>$ mannoside on acetonization passed into 2-3, 5-6 diacetone α -methyl $<1,4>$ mannoside. It was interesting to see whether α -methyl $<1,5>$ glucoside under similar conditions would preserve its lactal structure, or whether, similarly to the mannoside, it would change the size of its ring. It was found, however, that the reaction with α -methyl $<1,5>$ glucoside proceeds very slowly and that the reaction product consists chiefly of diacetone-glucose. The difference between the two reactions may be due to two factors; first, to the greater stability of the glucosidic group in the mannoside as compared with that in the glucoside, and, second, to the allocation in the hydroxyl groups on carbon atoms (2) and (3) of mannose.



¹ Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, 1928, lxxviii, 363.

Thus in the case of methylmannoside the first step in the reaction most probably is the acetonization in positions (2-3) which is followed by a change in the ring structure and this again is followed by the addition of a second acetone group in positions (5-6). In the case of glucose the first step in the reaction is either a hydrolysis of the methyl group or a direct acetonization with the elimination of the methyl group, which then leads to diacetone-glucose.

We then took this occasion to study the reaction of addition of acetone to γ -methylglucoside. 5,6-Acetone methyl $\langle 1,4 \rangle$ glucoside has in reality not been prepared in pure state. Macdonald² has prepared, starting with a product which he regarded as diacetalglucose, a substance which analyzed approximately for a methylmonoacetoneglucose. The product was undoubtedly impure. Later Irvine, Fyfe, and Hogg,³ on heating γ -methylglucoside with acetone, obtained a product which they employed for the preparation of 1,2,3-trimethyl-5,6-monoacetone glucose. The reaction product was a mixture of two substances. The lower boiling fraction supposedly consisted of a substance of the following structure (III).



This substance was regarded as an oxidation product of glucose. The oxidation supposedly occurred during the process of methylation.

5,6-Acetone methyl $\langle 1,4 \rangle$ glucoside is easily prepared by shaking methyl $\langle 1,4 \rangle$ glucoside with acetone in the presence of

² Macdonald, J. L. A., *J. Chem. Soc.*, 1913, ciii, 1896.

³ Irvine, J. C., Fyfe, A. W., and Hogg, T. P., *J. Chem. Soc.*, 1915, cvii, 524.

anhydrous copper sulfate at ordinary temperature. The product obtained in this manner is soluble in ether and in methyl iodide. It can be distilled and the product analyzes correctly for mono-acetone methylglucoside and is dextrorotatory. It can then be further methylated by means of methyl iodide and silver oxide. The product of this reaction is 2,3-dimethyl-5,6-acetone methyl $<1,4>$ glucoside. Under these conditions, no high boiling fraction is obtained. This substance also is dextrorotatory. We then repeated the acetonylation under the conditions given by Macdonald and obtained a substance very similar to his which, however, was not so pure and uniform a product as the one described above.

EXPERIMENTAL.

Experiments with Acetone on α -Methyl $<1,5>$ Glucoside.

1. *With Copper Sulfate.*—100 gm. of α -methylglucoside were shaken with 1000 cc. of acetone and 100 gm. of anhydrous copper sulfate at 40° for 48 hours. No α -methylglucoside was dissolved.

2. *With Acetone-Hydrochloric Acid.*—100 gm. of α -methylglucoside were shaken at room temperature with 1000 cc. of acetone containing 1 per cent of hydrochloric acid. The acetone-hydrochloric acid was changed daily until all of the glucoside had dissolved. The acetone solutions were neutralized with ammonia gas, filtered from inorganic salts, and concentrated under diminished pressure. The product soon partly crystallized. The crystals proved to be unchanged methylglucoside.

The product was taken up in a large quantity of dry ether, filtered, and concentrated to a syrup. In a short while the product crystallized. The crystals were filtered from the syrup through silk and recrystallized from ligroin ($80-90^\circ$). The substance had a m.p. of 108° and analyzed for diacetoneglucose. It contained no methoxyl.

3.690 mg. substance: 7.505 mg. CO_2 and 2.640 mg. H_2O .

$\text{C}_{13}\text{H}_{23}\text{O}_6$. Calculated. C 55.23, H 7.70.

Found. " 55.23, " 8.00.

The syrup which was separated from the crystals was fractionated. Aside from a low boiling condensation product of acetone the main fraction distilled at 148° , $p = 0.1$ mm., and soon crystallized on standing. It also proved to be diacetoneglucose.

Action of Acetone on γ -Methylglucoside.

γ -Methylglucoside was prepared following the directions of Fischer. 80 gm. of glucose were shaken 18 hours at 15° with 1500 cc. of methyl alcohol containing 1 per cent of hydrochloric acid. The sugar was completely dissolved. The acid was removed with silver carbonate and the filtrate concentrated under reduced pressure at low temperature. The γ -methylglucoside was extracted by means of ethyl acetate which was again removed under reduced pressure in bottles suitable for shaking. The syrup was reextracted with ethyl acetate and filtered from a small amount of α -glucoside. The ethyl acetate was again removed under diminished pressure. To complete the operation, acetone (200 cc.) was added to the residue and the distillation was repeated.

The syrup, which weighed about 40 gm., was shaken with 1000 cc. of acetone and 100 gm. of anhydrous copper sulfate at room temperature for 18 hours. The filtrate was concentrated under diminished pressure to a syrup. This syrup, which amounted to 30 gm., was almost completely soluble in ether. The filtered ether solution was concentrated under reduced pressure and the syrup fractionated. It distilled at 148° at 0.1 mm. At room temperature it is more viscous than glycerol. It is soluble in water, alcohol, ether, and acetone. The analysis was as follows:

3.835 mg. substance: 7.165 mg. CO₂ and 2.600 mg. H₂O.

0.1890 gm. " : 0.1786 gm. AgI.

C₁₀H₁₈O₆. Calculated. C 51.25, H 7.70, OCH₃ 13.25.

Found. " 50.94, " 7.58, " 12.47.

This monoacetone methylglucoside had the following optical rotation in methyl alcohol.

$$[\alpha]_D^{25} = \frac{+ 1.82^\circ \times 100}{1 \times 5.00} = + 36.4^\circ.$$

It does not reduce Fehling's solution until after hydrolysis. It is completely hydrolyzed by 0.1 N HCl in boiling water in 90 minutes. 250 mg. (equivalent to 207 mg. of methylglucoside) were heated with 5 cc. of 0.1 N HCl in boiling water for 90 minutes. The rotation then was

$$[\alpha]_D^{25} = \frac{+ 2.14^\circ \times 100}{1 \times 4.14} = + 51.7^\circ.$$

Dimethylmonoacetone Methylglucoside.—8 gm. of monoacetone methylglucoside were dissolved with gentle warming in 70 cc. of methyl iodide. It was methylated with 80 gm. of silver oxide and an additional 70 cc. of methyl iodide. The methylated product was isolated as usual, the yield amounting to 7 gm. It is a very mobile liquid distilling at 105° at 0.3 mm. pressure. It does not reduce Fehling's solution until hydrolyzed. The analysis was as follows:

7.010 mg. substance: 14.125 mg. CO₂ and 5.235 mg. H₂O.

0.1770 gm. " : 0.4564 gm. AgI.

C₁₁H₂₀O₄. Calculated. C 54.96, H 8.46, OCH₃ 35.5.

Found. " 54.94, " 8.35, " 34.03.

Dimethylmonoacetone methylglucoside has the following optical rotation in methyl alcohol.

$$[\alpha]_D^{25} = \frac{+ 0.25^\circ \times 100}{1 \times 6.65} = + 3.76^\circ.$$

Monoacetoneglucosidimethylacetal (?) of Macdonald.

The procedure as given by Macdonald was followed, except for the time of action of acetone-hydrochloric acid, which was shortened. 90 gm. of α -glucose were dissolved by being refluxed on a steam bath in 1100 cc. of methyl alcohol. When all the sugar had dissolved, the solution was cooled and 300 cc. of methyl alcohol containing 25 gm. of hydrochloric acid were added. In one instance the acid was allowed to act for 60 hours and in another for 24 hours. The results in both cases were the same. The acid was neutralized with dry barium carbonate, the solution was filtered, and then shaken with an excess of dry silver carbonate. The filtered solution was concentrated in bottles suitable for shaking. Acetone (about 200 cc.) was added and removed under reduced pressure to displace traces of methyl alcohol. The syrup was then shaken without heating with 3 portions of 500 cc. of acetone containing 0.5 per cent hydrochloric acid for 45 minutes for each portion. The acetone filtrates were neutralized with ammonia gas, combined, filtered, and concentrated under reduced pressure. The syrup which remained was extracted with ether and filtered. After removal of the ether under reduced pressure, 30 gm. of a

clear syrup were obtained. The syrup was repeatedly extracted with low boiling petroleic ether and finally taken up again in ether and concentrated to a syrup.

This product was heated at 60° for a considerable time at low pressure. It then had a rotation of

$$[\alpha]_D^{25} = \frac{-0.55^\circ \times 100}{1 \times 4.60} = -11.95^\circ.$$

The analysis was as follows:

3.320 mg. substance: 6.330 mg. CO₂ and 2.305 mg. H₂O.

0.1420 gm. " : 0.1149 gm. AgI.

C₁₀H₁₈O₆. Calculated. C 51.25, H 7.70, OCH₃ 13.25.

Found. " 51.99, " 7.76, " 10.68.

The product did not reduce Fehling's solution until after hydrolysis. It was completely hydrolyzed with 0.1 N HCl in 90 minutes. When subjected to fractional distillation it distilled at 148–150°, p = 0.1 mm., but it then became dextrorotatory (in one instance $[\alpha]_D^{25} = +20^\circ$, and in another $[\alpha]_D^{25} = +7.0^\circ$). A methoxy determination showed only 8.12 per cent OCH₃.

STUDIES IN POLYMERIZATION AND CONDENSATION.

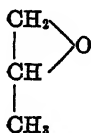
IV. EXPERIMENTS ON GLYCIDOL ACETATE.

By P. A. LEVENE AND A. WALTJ.

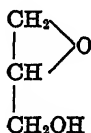
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New York.)

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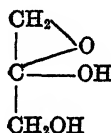
In previous papers of this series¹ the tendencies towards polymerization or towards condensation of the following substances were tested: propylene oxide (I), glycidol (II), and dihydroxyacetone (III). The first two substances contain an ethylene oxide radical in their molecules and the third may exist in the ethylene oxide form, as can be seen from the following formulæ.



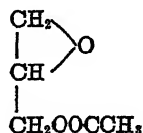
I.



II.

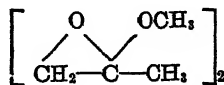
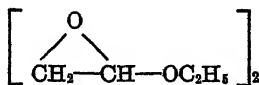


III.



IV.

Under the conditions described in the previous articles, these substances showed a tendency to condense into monomolecular structures consisting of two or more radicals of the parent substance. On the other hand, the half acetals of glyoxal and of acetol, according to Bergmann,² readily condense into the dimeric forms.



Our observations have now been extended to glycidol acetate (IV) and to acetol acetate.

¹ Levene, P. A., and Waltj, A., *J. Biol. Chem.*, 1927, lxxv, 325; 1928, lxxviii, 23.

² Bergmann, M., and Ludewig, S., *Ann. Chem.*, 1924, cdxxxvi, 173.

Glycidol acetate may be regarded as standing between propylene oxide and glycidol. Like the first, it has on carbon atom (3) no mobile hydrogen atom, and, like the second, it has an oxygen atom attached to carbon atom (3). The substance was allowed to stand for 5 days at 135–140° and then for several weeks at 12–13°. The product was then fractionated by distillation under reduced pressure. The distillation apparatus was so arranged that in the receiving flask a crystalline part could be collected separately from the liquid distillate. The distillate was collected in two receivers, the first cooled by running water; the vapors escaping the first receiver were collected in a U-tube immersed in a mixture of solid carbon dioxide and acetone.

The material obtained on distillation will be discussed in three parts. First, the distillate received in the main distilling flask at the temperature of 101–125° at 0.05 mm. pressure will be referred to as Distillate I. Second, the material obtained at the temperature of 120–130° and 0.05 mm. pressure will be referred to as Distillate II. Finally, the material received in the U-tube will be referred to as Distillate III.

Distillate I consisted of a crystalline fraction, Distillate I_1 , and of a liquid fraction, Distillate I_2 .

Distillate I_1 , which was crystalline at 0°, had the molecular weight of 226 which corresponds quite well to the dimeric form of glycidol acetate (the theory requires 232) and gave the correct saponification number. On hydrolysis, it yielded glycerol. Hence, there is no doubt that the crystalline material represented a pure polymerization product of glycidol acetate. At room temperature the crystals melted and on standing 1 week at room temperature the viscous substance turned semisolid. The product analyzed slightly low for glycidol acetate; namely, C = 51.19, H = 6.99, whereas the theory requires C = 51.73, H = 6.90. This material had a very high molecular weight, approximating 1450. On acetylation and subsequent distillation the material consisted chiefly of triacetin and of a small quantity of material boiling at 150–155° at 0.2 mm. pressure, which might have consisted of an acetylated condensation product of glycidol.

Distillate I_2 was separated into two parts. *Part A* was soluble in benzene. The material analyzed as follows: C = 48.51, H = 7.72. On hydrolysis of this substance the products were

glycerol and a dark colored residue. The fraction was a mixture of several substances of which one was either a polymer of glycidol acetate or a mixture of acetins and another was possibly a condensation product of glycidol. *Part B* was insoluble in benzene. From the solubility of the material and from the low carbon content, the conclusion is warranted that it consisted of glycerol not completely acetylated (monoacetin). Indeed, on acetylation of this material triacetin and a small proportion of tetraacetyldiglycerol were obtained.

Distillate II.—This fraction consisted of material which had a carbon content of 50.61 and a hydrogen content of 7.13. Its saponification value was 304. It was partly insoluble in benzene. Thus also this fraction consisted in part of incompletely acetylated glycerol and possibly of a condensation product. Indeed, on acetylation it yielded principally triacetin and a small proportion of a high boiling liquid.

Distillate III.—The material of this fraction on being cooled crystallized in part. On distillation of the acetylation material, the principal product was triacetin; a small portion of a high boiling substance also was formed. However, the carbon content of the original material was lower than that required by theory for glycidol acetate or for its polymer. It seems possible that a small portion of glycerol and of monoacetin was formed which subsequently condensed with glycidol acetate. Whether the condensation took place during distillation or prior to it remains uncertain.

From all the above observations it follows that glycidol acetate has a greater tendency to polymerize than propylene oxide, glycidol, or dihydroxyacetone; on the other hand, its tendency to form condensation products is much lower than that of the latter substances.

In contrast to glycidol acetate, acetol acetate (monohydroxyacetone acetate) shows no tendency to polymerization nor does it show a tendency towards condensation.

For convenience the composition of the various fractions obtained from glycidol acetate is presented in Tables I and II.

EXPERIMENTAL.

Glycidol acetate was obtained by the action of potassium acetate on epichlorohydrin. The material was fractionated three

TABLE I.

Polymerization of Glycidol Acetate.

<i>Distillate I₁</i> (crystalline).		<i>Distillate I₂</i> (liquid).		<i>Distillate II</i> (liquid).		<i>Distillate III</i> (crystalline).	
Remains crystalline at 0°; melts at laboratory temperature to a very viscous substance. Soluble in benzene. Mol. wt., calculated (dimeric).....232. Saponification No. calculated.....226. Saponification No. found.....494.		B.p. 101-125° at 0.05 mm. Saponification No. 327.		B.p. 120-130° at 0.05 mm. C 50.61, H 7.13. Saponification No. 304. Partly soluble in benzene.		(Substance in CO ₂ -cooled U-tube.) Soluble in benzene. C 50.65, H 6.69. Saponification No. 417.	
Standing 1 wk. Acid hydrolysis, 6 gm. Glycerol 2.1 gm.	Changes its consistency and becomes semisolid at room temperature. Mol. wt. 1450. Calculated. C 51.73, H 6.90. Found. " 51.19, " 6.99.	Extracted with benzene. Gives two fractions.		See Table II.			
		Triacetin 4.8 gm.		Acetylation 3.1 gm.		Triacetin 4.9 gm.	
		0.6 gm. substance. B.p. 150-155° at 0.2 mm Saponification No. 587. C 50.81, H 6.72.		1.6 gm. substance. B.p. 150-165° at 0.1-0.2 mm. Saponification No. 618. Mol. wt. 384.		0.7 gm. substance. B.p. 155-163° at 0.1 mm. Saponification No. 570	

TABLE II.
Fractionation of Distillate I₂ by Benzene Extraction.
 Distillate I₂.

Benzene extraction.							
<p>Part A. Benzene-soluble fraction. C 48.51, H 7.72. Saponification No. 422.</p> <p>4.9 gm. refluxed with 50 cc. of 1.0 N NaOH, neutralized, concentrated, extracted with acetone, and distilled.</p> <p>0.6 gm. substance. B.p. of glycerol (117–118° at 0.2 mm.). C 40.36, H 8.98. Dark-colored residue (0.4 gm.).</p>	<p>Part B. Benzene-insoluble fraction. Concentrated from methyl alcohol solution and distilled.</p> <table> <tr> <td> <p>Fraction 1. B.p. 103–112° at 0.2–0.3 mm. Not uniform (contained threads). Monoacetin. Calculated. C 44.75, H 7.52, saponification No. 418. Found. C 44.30, H 7.82, saponification No. 386.</p> </td><td> <p>Fraction 2 (0.7 gm.). B.p. 112–125° at 0.2 mm. C 46.36, H 7.68. Saponification No. 209.</p> </td></tr> <tr> <td> <p>Acetylation</p> </td><td> <p>Acetylation.</p> </td></tr> <tr> <td> <p>Acetylation 3.2 gm.</p> </td><td> <p>Triacetin 0.6 gm.</p> </td></tr> </table> <p>Triacetin. 5.0 gm. = 96 per cent yield if starting material was pure monoacetin.</p> <p>Residue 0.4 gm. Tetraacetyldiglycerol. Calculated. C 50.28, H 6.64, saponification No. 671. Found. C 50.67, H 6.96, saponification No. 631.</p>	<p>Fraction 1. B.p. 103–112° at 0.2–0.3 mm. Not uniform (contained threads). Monoacetin. Calculated. C 44.75, H 7.52, saponification No. 418. Found. C 44.30, H 7.82, saponification No. 386.</p>	<p>Fraction 2 (0.7 gm.). B.p. 112–125° at 0.2 mm. C 46.36, H 7.68. Saponification No. 209.</p>	<p>Acetylation</p>	<p>Acetylation.</p>	<p>Acetylation 3.2 gm.</p>	<p>Triacetin 0.6 gm.</p>
<p>Fraction 1. B.p. 103–112° at 0.2–0.3 mm. Not uniform (contained threads). Monoacetin. Calculated. C 44.75, H 7.52, saponification No. 418. Found. C 44.30, H 7.82, saponification No. 386.</p>	<p>Fraction 2 (0.7 gm.). B.p. 112–125° at 0.2 mm. C 46.36, H 7.68. Saponification No. 209.</p>						
<p>Acetylation</p>	<p>Acetylation.</p>						
<p>Acetylation 3.2 gm.</p>	<p>Triacetin 0.6 gm.</p>						

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times, with the aid of a small distilling column, under reduced pressure, and finally at ordinary pressure. It distilled at 162–164° at 750 mm. The analysis was as follows:

5.695 mg. substance: 10.750 mg. CO₂ and 3.555 mg. H₂O.

C₈H₈O₃. Calculated. C 51.73, H 6.89.

Found. " 51.47, " 6.99.

51 gm. of this substance were placed in a round bottom flask which was connected with a reflux condenser carrying a calcium chloride tube. The flask was kept 5 days at 132–145°.

TABLE III.
Data on Comparison Compounds.

Substance.	Saponification No.	C	H	Molecular weight.
Glycidol acetate.....	484	51.73	6.89	116
Diacetylglycerylglycidol.....	484	51.73	6.89	232
Dimeric glycidol acetate.....	484	51.73	6.89	232
Glycerol.....	None.	39.13	8.67	92
Monoacetin.....	418	44.75	7.52	134
Diacetin.....	637	47.70	6.87	176
Triacetin.....	772	49.54	6.42	218
Monoacetyldiglycerol.....	270	46.13	7.74	208
Diacetyldiglycerol.....	449	47.98	7.25	250
Triacetyldiglycerol.....	576	49.30	6.90	292
Tetraacetyldiglycerol.....	671	50.28	6.64	334

The distillation apparatus was arranged in the following way. Into the main receiver was inserted a small vial in such a way as to collect the liquid part of the distillate while the vapors could condense on the walls of the main receiving flask. This flask was cooled with cold tap water. The vapors which escaped condensation in the first receiving flask were cooled in a U-tube which was immersed in a mixture of solid carbon dioxide and acetone.

At 35–45° and 1 mm. pressure, 10 cc. of liquid came over, and a smaller fraction at 55–105° at 0.3 mm. pressure. These fractions were not analyzed.

Distillate I was collected in the temperature interval of 101–125° at 0.05 mm. pressure. During this interval the liquid part was received in the vial (Distillate I₂), while the larger part settled on

the walls of the receiving flask in the form of beautiful crystals (Distillate I₁).

Distillate II was collected in the interval of 120–130° at 0.05 mm. pressure. It consisted of a fairly thick liquid. The crystalline material continued to settle on the walls of the receiving flask.

Distillate III was collected in the U-tube. The material of this fraction also had a crystalline character so long as the temperature was maintained below 0°.

These fractions will be discussed separately.

Distillate I.

Distillate I₁.

The main fraction of the distillate was the substance which condensed in crystalline form on the walls of the water-cooled receiving flask. At ordinary temperature the crystals melted, forming a viscous fluid. This fraction was soluble in benzene.

Saponification Number.—0.1494 gm. of substance was refluxed with 30 cc. of 0.1 N KOH for 1½ hours. 16.8 cc. of 0.1 N HCl were required for neutralization. Saponification number, calculated 484, found 495.

Molecular Weight Determination by Method of Freezing Point Depression.—15.333 gm. benzene.

0.2688 gm. substance: 0.393° depression; molecular weight 226.5.
0.4778 “ “ : 0.688° “ “ “ 230.0.

For (C₅H₈O₃)₂ molecular weight calculated 232.

Hydrolysis of the Substance.—6 gm. of the substance were refluxed 5 hours with 45 cc. of 5 per cent sulfuric acid. The acetic acid thus formed was extracted by means of ether in a continuous extraction apparatus. The operation was continued for 15 hours. From the remaining aqueous solution the sulfuric acid was removed quantitatively by means of a solution of barium hydroxide. The filtrate was then concentrated under reduced pressure and the residue distilled at 112–114° at 0.05 mm. Yield 2.1 gm. The substance had the composition of glycerol.

3.815 mg. substance: 5.385 mg. CO₂ and 3.025 mg. H₂O.

C₅H₈O₃. Calculated. C 39.13, H 8.67.

Found. “ 38.49, “ 8.87.

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Thus it is evident that the material consisted of practically pure dimeric glycidol acetate.

Further Polymerization of Distillate I₁.

After it had stood 1 week at room temperature the viscosity of the substance was greatly increased. It had the following composition.

5.500 mg. substance: 10.305 mg. CO₂ and 3.400 mg. H₂O.

C₈H₈O₃. Calculated. C 51.73, H 6.89.

Found. " 51.19, " 6.91.

Molecular Weight Determination of the More Viscous Substance by Method of Menzies and Wright.—32.0 cc. of benzene (b.p. 79.9° at 750 mm.).

Weight of substance.	Elevation on differential thermometer.	Molecular weight.
gm.	mm.	
0.2463	3.5	1411
0.3975	5.5	1450

Acetylation of This Material.—To 3.1 gm. of the substance which had become semisolid on standing for a week were added 29 gm. of freshly distilled acetic anhydride and the mixture was refluxed for 1½ hours. The excess of the anhydride was removed under diminished pressure. Two fractions were obtained.

A. 86–87° at 0.05 mm., 4.8 gm.

B. 150–155° " 0.2 " 0.6 "

Fraction A.—This fraction had the composition of triacetin.

4.310 mg. substance: 7.895 mg. CO₂ and 2.605 mg. H₂O.

C₈H₈(OCOCH₃)₃. Calculated. C 49.54, H 6.42.

Found. " 49.99, " 6.75.

Saponification Number.—0.1565 gm. of substance was refluxed for 1½ hours with 40.0 cc. of 0.1 N KOH. 14.2 cc. of 0.1 N HCl were required for neutralization. Saponification number, calculated 772, found 776.

Molecular Weight Determination by Method of Menzies and Wright.—32.0 cc. of benzene; b.p. 80.1° (at 756 mm.).

Weight of substance.	Elevation on differential thermometer.	Molecular weight.
<i>gm.</i>	<i>mm.</i>	
0.5137	46	226
0.6799	62	221

Molecular weight calculated 218.1.

Fraction B.—This fraction had the following composition.

4.465 mg. substance: 8.320 mg. CO₂ and 2.685 mg. H₂O.

Found. C 50.81, H 6.72.

Saponification Number.—0.1041 gm. of substance was refluxed for 1½ hours with 30.0 cc. of 0.1 N KOH. 19.1 cc. of 0.1 N HCl were required for neutralization. Saponification number, found 587.

Thus, it is seen that on standing the dimeric polymer of glycidol acetate undergoes further polymerization giving rise to a substance of high molecular weight which on acetylation is transformed into triacetin. It is possible that a small proportion of condensation product also is formed.

Distillate I₂.

This distillate was extracted with benzene at room temperature and each of the two fractions was treated separately.

Fraction A, Soluble in Benzene.—The benzene was removed by distillation under a pressure of 15 mm. and finally at 1.0 mm. The extract had the following composition.

3.805 mg. substance: 6.770 mg. CO₂ and 2.630 mg. H₂O.

Found. C 48.51, H 7.72.

Saponification Number.—4.9 gm. of this material were refluxed with 50 cc. of 1.0 N NaOH. 13.1 cc. of 1.0 N HCl were required for neutralization. Saponification number, found 422.

The mixture was concentrated under diminished pressure. At

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a temperature of 117–118° at 0.2–0.3 mm., 0.6 gm. of substance distilled, apparently being impure glycerol.

4.405 mg. substance: 6.520 mg. CO₂ and 3.535 mg. H₂O.

C₈H₈(OH)₃. Calculated. C 39.13, H 8.69.

Found. " 40.36, " 8.98.

This fraction, then, contained a considerable part of either acetins or of a polymeric form of glycidol acetate and perhaps some condensation product.

Fraction B, Insoluble in Benzene.—This fraction was taken up in dry methyl alcohol, concentrated under diminished pressure, and distilled. Two fractions were obtained by distillation.

1. 103–112° at 0.2–0.3 mm., 3.6 gm.

2. 112–125° " 0.2 mm., 0.7 "

Fraction 1.—During the latter part of the distillation of this fraction, formation of threads was observed in the distillate. This was probably due to a small amount of glycerol. The fraction had the composition of monoacetin.

5.340 mg. substance: 8.675 mg. CO₂ and 3.735 mg. H₂O.

C₈H₇O₂(OCOCH₃). Calculated. C 44.75, H 7.52.

Found. " 44.30, " 7.82.

Saponification Number.—0.1284 gm. of substance was refluxed for 1½ hours with 30.0 cc. of 0.1 N KOH. 21.2 cc. of 0.1 N HCl were required for neutralization. Saponification number, calculated 418, found 386.

Acetylation of Fraction 1.—3.2 gm. of this substance were refluxed for 1½ hours with 23 gm. of freshly distilled acetic anhydride. The mixture was concentrated under reduced pressure. 5.0 gm. of substance distilling at 85–87° at 0.1 mm. gave a saponification number corresponding to triacetin.

Saponification Number.—0.1775 gm. of substance was refluxed with 35.0 cc. of 0.1 N KOH for 1½ hours. 10.9 cc. of 0.1 N HCl were required for neutralization. Saponification number, calculated 772, found 762.

Fraction 2.—This smaller fraction had the following composition.

6.690 mg. substance: 11.375 mg. CO₂ and 4.595 mg. H₂O.

Found. C 46.36, H 7.68.

Saponification Number.—0.1288 gm. of substance was refluxed for $1\frac{1}{2}$ hours with 31.0 cc. of 0.1 N KOH. 26.2 cc. of 0.1 N HCl were required for neutralization. Saponification number, found 209.

Acetylation of Fraction 2.—0.55 gm. of this substance was refluxed for $1\frac{1}{2}$ hours with 5 gm. of freshly distilled acetic anhydride. The mixture was concentrated under diminished pressure. 0.6 gm. of substance distilled at a temperature corresponding to triacetin.

Saponification Number.—0.1339 gm. of substance was refluxed for $1\frac{1}{2}$ hours with 30.0 cc. of 0.1 N NaOH. 12.6 cc. of 0.1 N HCl were required for neutralization. Saponification number, calculated 772, found 730.

The residue from the acetylation of Fraction 2 (0.4 gm.) gave the following results for tetraacetyldiglycerol.

3.630 mg. substance: 6.745 mg. CO_2 and 2.260 mg. H_2O .

$\text{C}_{14}\text{H}_{22}\text{O}_8$. Calculated. C 50.28, H 6.64.

Found. " 50.67, " 6.96.

Saponification Number.—0.0916 gm. of substance was refluxed for $1\frac{1}{2}$ hours with 35.0 cc. of 0.1 N KOH. 24.7 cc. of 0.1 N HCl were required for neutralization. Saponification number, calculated 671, found 631.

Thus the benzene-insoluble part of Distillate I_2 consisted of monoacetin and of a smaller portion of partly acetylated diglycerol.

Distillate II.

This fraction was collected in the receiving flask at the temperature 120–130°. It was a liquid, partly soluble in benzene.

Acetylation of Distillate II.—To 3 gm. of this material were added 22 gm. of freshly distilled acetic anhydride and the mixture was refluxed for 2 hours. The following two fractions were obtained.

A. 98–100° at 0.4 mm., 3.1 gm.

B. 150–165° " 0.1–0.2 mm., 1.6 gm.

Fraction A.—This substance had the composition of triacetin.

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5.000 mg. substance: 9.075 mg. CO₂ and 2.925 mg. H₂O.

C₅H₅(OCOCH₃)₃. Calculated. C 49.54, H 6.42.

Found. " 49.49, " 6.54.

Saponification Number.—0.1694 gm. of substance was refluxed for 1½ hours with 35.0 cc. of 0.1 N KOH. 11.2 cc. of 0.1 N HCl were required for neutralization. Saponification number, calculated 772, found 788.

Molecular Weight Determination by Method of Menzies and Wright.—32.0 cc. of benzene; b.p. 80.1° (at 756 mm.).

Weight of substance.	Elevation on differential thermometer.	Molecular weight.
gm.	mm.	
0.2923	27	220
0.4262	39	221

Molecular weight calculated 218.1.

Fraction B.—This material had the following composition.

5.135 mg. substance: 9.615 mg. CO₂ and 3.085 mg. H₂O.

Found. C 51.06, H 6.72.

Saponification Number.—0.1665 gm. of substance was refluxed for 1½ hours with 30.0 cc. of 0.1 N KOH. 11.65 cc. of 0.1 N HCl were required for neutralization. Saponification number, found 618.

Molecular Weight Determination by Method of Menzies and Wright.—32.0 cc. of benzene; b.p. 80.1° (at 756 mm.).

Weight of substance.	Elevation on differential thermometer.	Molecular weight.
gm.	mm.	
0.4993	26	386
0.6824	36	383
0.8778	46	385

The facts that the substance was only partly soluble in benzene, that the greater part of it was converted into triacetin, and that after acetylation a small fraction distilled at 150–165°, indicate that the greater part of the material consisted of partly

acetylated glycerol and that a small part may have consisted of the polymer and of a higher condensation product.

Distillate III.

The vapors escaping from the main receiving flask passed through 1 foot of rubber tubing before they reached the well cooled U-tube. The contents of the U-tube were crystalline in part. The U-tube was heated to transfer the substance into a small flask. The substance had the following composition.

3.685 mg. substance: 6.845 mg. CO_2 and 2.205 mg. H_2O .

Found. C 50.65, H 6.69.

Saponification Number.—0.1398 gm. of substance was refluxed with 25 cc. of 0.1 N KOH for $1\frac{1}{2}$ hours. 14.6 cc. of 0.1 N HCl were required for neutralization. Saponification number, found 417.

Molecular Weight Determination by Method of Freezing Point Depression.—12.016 gm. benzene.

0.2162 gm. substance; 0.145° freezing point depression. Found molecular weight, 630.

0.4454 gm. substance; 0.279° freezing point depression. Found molecular weight, 675.

Acetylation of Distillate III.—To 3.5 gm. of this substance were added 22 gm. of freshly distilled acetic anhydride and the mixture was refluxed for $1\frac{1}{2}$ hours. The excess of acetic anhydride was removed under diminished pressure. Two fractions were obtained.

A. 100–101° at 0.5 mm., 4.9 gm.

B. 155–163° " 0.1 " 0.7 "

Thus, this fraction consisted in the main of a polymer of glycidol acetate, inasmuch as it had a high molecular weight and on acetylation yielded principally triacetin. On the other hand, the facts that the carbon content was low for glycidol acetate, and that a high boiling fraction was obtained on distillation of the acetylation product, indicate the formation of a small proportion of a condensation product.

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Experiment with Acetol Acetate.

The acetol acetate was obtained by heating bromoacetone with dry potassium acetate in absolute alcohol. The redistilled acetate boiled at 170–171° at 755 mm.

28 gm. of this substance were heated under the same conditions for the same length of time as glycidol acetate. On distillation under reduced pressure at 15–17 mm., 26.5 gm. of unchanged acetol acetate were recovered. The small amount of residue was dark and more viscous than the original acetol acetate. It had the following composition.

3.660 mg. substance: 6.950 mg. CO₂ and 2.280 mg. H₂O.

OC₃H₅OCOCH₃. Calculated. C 51.73, H 6.89.

Found. " 51.77, " 6.97.

THE BASIC AMINO ACIDS OF HORSE HEMOGLOBIN.*

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New Haven.)

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Although the physicochemical properties of different hemoglobins have received a great deal of attention in recent years, surprisingly little work has been done upon the quantitative isolation of amino acids from these important proteins. Abderhalden in 1903 (1) carried out a complete analysis of horse hemoglobin which accounted for about two-thirds of the molecule, and Abderhalden and Baumann (2) in 1907 investigated the mono-amino acids of dog hemoglobin; but since then there have been no comprehensive attempts made, so far as we can learn, to estimate the amino acid constituents of hemoglobins by direct isolation methods. We have therefore carried out an analysis of the basic amino acids of horse hemoglobin employing certain of the modifications of the procedure of Kossel that have been described in a recent paper (3).

Abderhalden reported 10.5 per cent of histidine, 5.2 per cent of arginine, and 4.1 per cent of lysine isolated from this protein and these results have been widely quoted. His analysis was made upon a fraction obtained by precipitation with phosphotungstic acid from a solution of the amino acids, the esters of which had distilled between 130–160°. The accuracy of these figures depends upon the assumptions that esterification was complete, liberation of the esters, distillation, and subsequent hydrolysis of the esters took place without loss, and that precipitation by phosphotungstic acid was complete. When the well known difficulties of the Kossel

* The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

analysis are considered in addition to the improbability that all of these assumptions are justified, it is clear that these determinations may be somewhat in error. It may further be noted that some years later Abderhalden and Medigreceanu (4) reported only 5.3 per cent of histidine obtained from the red blood cells of the horse.

Van Slyke (5) in 1911 carried out an analysis of ox hemoglobin by his indirect method. Assuming that the material contained 16.8 per cent of nitrogen, his figures indicate the presence of 7.9 per cent of histidine, 4.0 per cent of arginine, and 9.5 per cent of lysine in this protein.

Hunter and Borsook (6) employed Van Slyke's method in the analysis of globin prepared from horse hemoglobin. Assuming that 2.1 per cent of the nitrogen of horse hemoglobin belongs to hematin and that hemoglobin contains 16.86 per cent of nitrogen, their results indicate that this protein yields 7.74 per cent of histidine, 4.11 per cent of arginine, and 9.55 per cent of lysine, figures which are in remarkably close agreement with Van Slyke's obtained from a different hemoglobin. These investigators also found 2.56 per cent of tryptophane and 4.53 per cent of tyrosine in this protein by the colorimetric methods of Folin and Looney. Hanke and Koessler (7), using their colorimetric procedure, have reported 8.9 per cent and 8.65 per cent of histidine in two different samples of horse hemoglobin.

Modifications of Method of Analysis.

Preliminary experiments upon a preparation of washed and coagulated blood cells indicated that certain changes in the procedure employed in the analysis of edestin (3) were necessary in the case of a protein containing a high proportion of histidine but a low proportion of arginine. It was found that, when present in high concentration, the silver compound of histidine may begin to precipitate at reactions decidedly acid to Congo red and, further, that precipitation was not entirely complete at pH 7.0. The silver chloride obtained upon removal of the hydrochloric acid was, therefore, thoroughly extracted with hot dilute hydrochloric acid and the removal of chloride repeated on the extract.

To insure complete precipitation of the histidine it was necessary to carry the reaction over to pH 7.4. Although previous investiga-

tion showed that this was probably quite safe (8), we felt it necessary to examine the filtrate from the mercuric sulfate precipitate of the histidine for arginine. A small amount was indeed found indicating either that a little arginine had been precipitated or that the reprecipitation of the histidine fraction, which is always practiced in order to avoid washing the voluminous precipitate, had failed of its object. We incline to the latter view.

In order to avoid as far as possible any loss of arginine due to the solubility of arginine silver each solution from which this substance had been precipitated was acidified, the barium sulfate was removed, and the solution concentrated to a small volume. A silver precipitate was then produced at alkaline reaction in the more concentrated solution and the material so obtained added to the main precipitate.

A further difficulty was encountered which may owe its origin to a small solubility of histidine silver. When the different precipitates of arginine silver were combined and decomposed the solution showed evidences of the presence of histidine. It was therefore necessary to repeat the silver separation on the main arginine fraction, this time at pH 7.2, and add the small histidine silver precipitate produced to the main portion. The arginine was then precipitated as its silver compound at alkaline reaction, the filtrate was concentrated, and the arginine precipitation repeated in small volume.

These changes in the procedure led to a somewhat complex scheme of fractionation. It seemed necessary, however, to leave no possibility of loss unexamined and to face the fact that the flocculent precipitates of these silver compounds can be adequately washed only by decomposition and subsequent reprecipitation.

The lysine fraction, obtained by means of phosphotungstic acid from the filtrate from the arginine and histidine silver precipitation, gave no difficulty. The mother liquors from the lysine picrate were combined and worked over with phosphotungstic acid until no further lysine could be obtained from them.

Bases of Horse Hemoglobin.

The results of the analysis are shown in Table I. It is clear that the proportion of histidine isolated agrees with the findings of Van Slyke and of Hunter and Borsook very closely and it would

therefore seem that the histidine content of this protein is now fairly well established. As in the edestin analysis we have been unable to check the colorimetric histidine values found by Hanke and Koessler.

Our figure for arginine is appreciably lower than that of Hunter and Borsook. This can be explained, in part, by the difficulty

TABLE I.
Bases of Horse Hemoglobin. First Analysis (200 Gm.).

The results are expressed as proportions of the free base in the protein as calculated from the weight of nitrogen or of salt.

	From weight of N.	From weight of salt.
	<i>per cent</i>	<i>per cent</i>
Histidine.		
N in main fraction.....	7.80	
Dinitronaphtholsulfonate from main fraction.....		7.57
Crude free histidine from main fraction.....	7.53	
Sulfonate from filtrate from HgSO ₄ precipitate....		0.06
“ “ main arginine fraction.....		0.005
		7.64
Arginine.		
N in main fraction.....	3.33	
Dinitronaphtholsulfonate from main fraction.....		2.99
Sulfonate from filtrate from HgSO ₄ precipitate....		0.12
		3.11
Lysine.		
N in main fraction.....	13.12	
Picrate from main fraction.....		6.20
“ “ working over mother liquors.....		1.89
		8.09

of determining arginine by Van Slyke's method in the presence of a large proportion of histidine. As Plimmer (9)¹ has shown, histidine is not entirely stable when heated with strong alkali, as much as 3 per cent of its nitrogen being split off as ammonia under the conditions of the Van Slyke estimation of arginine. This

¹ See also Vickery and Leavenworth (8).

would tend to give high results for arginine in the case of hemoglobin, which contains so large a proportion of histidine, although in most cases it could be safely disregarded. While this source of error is scarcely sufficient to account for all of the discrepancy between our results and those of Hunter and Borsook, it indicates a possibility that their figure may be too high.

As we have pointed out in a previous paper there is little ground for discussion of the true worth of lysine determinations by either the direct or the indirect methods. The direct method gives results which are probably too low, the indirect undoubtedly gives results which are too high.

Second Analysis of Hemoglobin.

In order to obtain further evidence regarding the proportion of arginine in horse hemoglobin a second analysis was carried out upon a smaller quantity of the preparation (97.45 gm.) by a simplified procedure. Hydrolysis was effected by boiling for 38 hours with 8 N sulfuric acid. The greater part of the acid was removed and arginine and histidine were precipitated together as usual, the filtrate was acidified, concentrated, and a second alkaline silver precipitate taken out at a small volume. The two precipitates were combined and decomposed with hydrogen sulfide. The histidine was then precipitated by means of Hopkins' reagent and the filtrate, after removal of mercury and most of the sulfuric acid, was subjected to a silver precipitation at pH 7.2 to remove any possible histidine. The arginine was then precipitated at alkaline reaction. The histidine and arginine fractions were worked up in the usual way.

The dinitronaphtholsulfonates obtained from the main histidine and arginine fractions indicated the presence of 7.35 per cent and 3.32 per cent respectively of these two bases in hemoglobin, results which are in close agreement with the previous analysis and which confirm their order of magnitude. No histidine was found in the small silver precipitate obtained at pH 7.2 when working over the filtrate from the Hopkins' reagent precipitate for arginine.

Requirements of Theory.

The proportions of histidine, arginine, and lysine in hemoglobin are too high to have significance for the calculation of possible

values of the minimal molecular weight. Moreover, in this particular protein the use of these figures is unnecessary since the order of magnitude of the minimal molecular weight has been known with great accuracy since 1886 when Zinoffsky (10) carried out his analyses of iron and sulfur. The iron content (0.335 per cent) leads to the figure 16,670 and the sulfur content (0.390 per cent) to 8220. Zinoffsky calculated a formula for hemoglobin which contained 1 iron and 2 sulfur atoms and which implied a molecular weight of 16,710. The recent ultracentrifuge experiments of Svedberg and Fahraeus (11) and the osmotic pressure

TABLE II.

Proportions of Basic Amino Acids in Hemoglobin Calculated upon a Molecular Weight of 66,800 for This Protein.

		Calculated.	Found.
	<i>mols</i>	<i>per cent</i>	<i>per cent</i>
Histidine.	32	7.43	7.64
	33	7.66	
	34	7.89	
Arginine.	11	2.86	3.11
	12	3.13	
	13	3.39	
	14	3.64	
Lysine.	36	7.87	8.10
	37	8.09	
	38	8.30	

measurements of Adair (12) leave little doubt that the true molecular weight of hemoglobin lies very close to 66,800 and that the molecule therefore contains 4 atoms of iron and 8 of sulfur.

Although no final conclusions can be drawn from the present analysis it may be of some interest to point out the possibilities of molecular composition that are indicated. In Table II are presented calculations which show that our highest analytical figures are in closest agreement with the assumptions that the hemoglobin molecule yields 33 molecules of histidine, 13 of arginine, and 37 of lysine. Since they are derived from analytical results based upon isolation, these figures are probably minimal

values. Inasmuch as the molecular proportions are not divisible by four this result further suggests that the hemoglobin molecule is not formed of four symmetrical parts.

EXPERIMENTAL.

Hemoglobin.—The hemoglobin was prepared from 4.2 liters of centrifuged horse blood cells and crystallized twice from phosphate buffers by Dr. Arda A. Green of Dr. E. J. Cohn's laboratory at the Harvard Medical School (13). We wish to express our thanks to Dr. Green and Dr. Cohn for the extraordinarily fine preparation. The ice-cold pulp was slowly poured into a large volume of boiling distilled water containing a trace of sodium chloride. The coagulum was strained off on cloth, suspended in cold water, and filtered. It was then ground in a plate mill, suspended in warm water, filtered, dried, and ground to a fine powder. The last wash water contained no chloride or phosphate. Yield 660 gm.; N, 16.69 per cent moisture-free; ash, 0.64 per cent.

Preparation of Hydrolysate.—Somewhat over 200 gm. of this preparation were boiled with 1800 cc. of 22 per cent hydrochloric acid for 30 hours, concentrated to a sirup *in vacuo*, diluted, and again concentrated three successive times.

The sirup was diluted and an aliquot part analyzed for nitrogen. The portion carried on for the analysis contained the equivalent of 199.5 gm. of the protein. It was diluted to approximately 6 liters and dilute sulfuric acid and silver oxide slowly added alternately with rapid stirring until the chloride was precipitated. The reaction was maintained acid to brom-phenol blue throughout. The silver chloride was removed and boiled with water containing a small excess of hydrochloric acid and centrifuged. This operation was repeated and the extracts were combined and treated with silver oxide and sulfuric acid as before. The second silver chloride precipitate was washed and the two solutions combined and concentrated *in vacuo* to about 6 liters and filtered perfectly clear.

Main Fractionation.—The hydrolysate was treated with silver oxide and sulfuric acid alternately until a positive test for excess silver was obtained,² and the solution was then made strongly

² We have occasionally encountered difficulty in obtaining this test. The trouble appears to be due to the too liberal addition of sulfuric acid, as we find that if the acidity is reduced until such a solution is only faintly

alkaline to phenolphthalein by the addition of warm saturated barium hydroxide solution. The precipitate ($H_1 + A_1$) was filtered off and, without washing, suspended in water to which a slight excess of sulfuric acid was added. The filtrate (10 liters) was acidified, barium sulfate was removed and washed, the solution was concentrated to about 2 liters, excess silver was added, and a second precipitate ($H_2 + A_2$) was taken out at strongly alkaline reaction and added to the first. The precipitates were then treated with hydrogen sulfide on a shaking machine. The filtrate was diluted, acidified, treated with hydrogen sulfide, the precipitate removed and washed, and the filtrate (L_1) set aside for the subsequent precipitation of the lysine.

The decomposed silver precipitates were freed from silver sulfide, concentrated to about 3.5 liters, and excess silver added as before. The solution was then brought to pH 7.4, centrifuged, the precipitate (H_3) suspended in acidified water and decomposed with hydrogen sulfide. The filtrate (7.2 liters) was made alkaline to phenolphthalein, the precipitate (A_3) of arginine silver removed, the filtrate acidified, concentrated, and the alkaline silver precipitation repeated in a volume of 1.5 liters. The filtrate from this precipitate (A_4) was freed from reagents and added to the lysine fraction as L_2 .

It is unnecessary to describe in detail the further steps taken to insure the complete separation and purity of the histidine and arginine fractions. In brief the histidine fraction, H_3 , was again subjected to a silver precipitation at pH 7.4, whereby H_4 was secured and the filtrate from this yielded successive arginine fractions, A_5 and A_6 , and a filtrate, L_3 , for the lysine fraction. All of the arginine fractions were combined and the silver separation repeated obtaining H_5 , which was added to H_4 , and A_7 and A_8 , which contained the whole of the arginine except the small amount which might still be retained by the voluminous histidine fraction H_4 . The filtrate L_4 was combined with the lysine fractions.

acid to brom-phenol blue the test can be readily obtained provided excess silver is present. The statement on p. 711 of our earlier paper (3) to the effect that a liberal amount of sulfuric acid should be added at the beginning is therefore misleading. The acidity should not be allowed to become unduly great.

Histidine Fraction.—The precipitates H_4 and H_5 were decomposed and the solution brought to a volume of about 2 liters. Sulfuric acid to make 5 per cent was added and the histidine was precipitated by the addition of 3 liters of Hopkins' reagent. The precipitate, which was filtered off after standing 2 days, was decomposed with hydrogen sulfide and the filtrate from the mercuric sulfide brought to pH 5 to 6 by means of barium hydroxide and filtered. The solution was made to 2000 cc. It contained 4.218 gm. of nitrogen, the equivalent of 15.56 gm. of histidine or 7.80 per cent of the protein. Histidine was determined in the usual way with dinitronaphtholsulfonic acid in two 200 cc. aliquots. The average indicated that the whole solution contained 15.10 gm. of histidine equivalent to 7.57 per cent of the protein. The histidine dinitronaphtholsulfonate contained 8.18 per cent of sulfur, theory 8.17 per cent.

As further evidence of the purity of this fraction, the remaining 1580 cc. were brought to pH 7.2, the approximate isoelectric point of histidine, by means of barium hydroxide. The solution was filtered and concentrated *in vacuo* to about 100 cc., heated until the separating histidine had redissolved, and 200 cc. of absolute alcohol were added. On being chilled overnight 10.38 gm. of well crystallized and fairly pure histidine separated (nitrogen found, 26.4 per cent, calculated, 27.10 per cent). The mother liquor, when concentrated to 15 cc. and treated with 2 volumes of alcohol as before, yielded a further 1.19 gm. of somewhat less pure (nitrogen 26.1 per cent) but well crystallized histidine. On the assumption that all the impurity in this histidine was due to tyrosine (14), the final mother liquor being disregarded, these two crops together account for 90 per cent of the calculated histidine of the solution.

Arginine Fraction.—The arginine precipitates A_7 and A_8 were combined, decomposed with hydrogen sulfide in the presence of a slight excess of sulfuric acid, freed from silver sulfide, and brought to 1000 cc. Analysis of 25 cc. aliquots of this solution indicated the presence of 2.136 gm. of nitrogen equivalent to 6.64 gm. of arginine or 3.33 per cent of the protein. The remaining 950 cc. were treated with sufficient barium hydroxide to reduce the acidity to approximately pH 5, barium sulfate was removed, and a slight excess over the theoretical amount of dinitronaphtholsul-

fonic acid was added to the hot solution at a volume of 800 cc. When the mixture stood overnight, 15.61 gm. of extremely fine arginine dinitronaphtholsulfonate separated, equivalent to 5.86 gm. of arginine for the whole solution or 88.2 per cent of the amount calculated from the nitrogen. This material contained 6.69 per cent sulfur, theory 6.56 per cent. The mother liquor was freed from reagents and subjected to a silver fractionation. An insignificant amount of material was precipitated at pH 7.2 and from this precipitate, H₆, 0.011 gm. of histidine or 0.005 per cent of the protein was isolated by means of dinitronaphtholsulfonic acid. The arginine precipitate, A₃, gave arginine dinitronaphtholsulfonate weighing 0.305 gm. equivalent to 0.115 gm. of arginine when corrected for the aliquots removed. Together these two crops of dinitronaphtholsulfonate account for 5.972 gm. of arginine. This is equivalent to 2.99 per cent of the protein.

Filtrate from Mercuric Sulfate Precipitate.—The filtrate from the Hopkins' reagent precipitate was freed from mercury and the greater part of the sulfuric acid, treated with an excess of silver oxide, and a histidine precipitate H₇ removed at pH 7.4 and an arginine precipitate A₁₀ at alkaline reaction. The histidine silver precipitate was decomposed and the histidine precipitated by Hopkins' reagent at a volume of 300 cc. The solution became faintly turbid within an hour and on standing 3 days a small precipitate separated. From this 0.124 gm. of histidine, equivalent to a further 0.06 per cent of the protein, was obtained as dinitronaphtholsulfonate by the usual procedure. This experience indicates that the precipitation of histidine by means of Hopkins' reagent is extraordinarily complete even in the large volume employed.

The arginine precipitate A₁₀ probably represents that part of the arginine which we had failed to wash out of the early histidine precipitates. After decomposition with hydrochloric acid 0.676 gm. of arginine dinitronaphtholsulfonate was secured. This is equivalent to 0.241 gm. of arginine or an additional 0.12 per cent of the protein.

Lysine Fraction.—The four solutions, L₁, L₂, L₃, and L₄, were combined and freed from silver, made alkaline with barium hydroxide, and concentrated *in vacuo* with the occasional addition of alcohol until the ammonia was removed. The solution was

then freed from barium and brought to a volume of 3 liters, 5 per cent of sulfuric acid was added, and the lysine was precipitated by the addition of 3 liters of 20 per cent phosphotungstic acid reagent. The precipitate was washed twice with dilute reagent, dissolved in 6 liters of 50 per cent acetone, and the phosphotungstic acid precipitated by barium hydroxide. The precipitate was thoroughly washed, first with cold and then with boiling alkaline water, and the clear solution was faintly acidified, concentrated, and all reagents were removed. Analysis of the solution showed the presence of 5.027 gm. of nitrogen. The crude lysine picrate secured in the customary way was recrystallized, two successive crops containing 11.40 and 0.97 gm., corrected, of lysine picrate being obtained. Both decomposed at 265–266° and were pure. This amount corresponds to 6.20 per cent of lysine in hemoglobin. All mother liquors were combined, picric acid was removed, and the basic substances were again precipitated with phosphotungstic acid. From this material lysine picrate decomposing at 261°, containing 3.78 gm. equivalent to a further 1.89 per cent of lysine, was secured. The filtrate from this crop was again worked over with phosphotungstic acid but no lysine picrate could be obtained from it.

SUMMARY.

Analysis of the basic amino acids of crystallized horse hemoglobin by direct isolation methods indicates that this protein yields 7.64 per cent of histidine, 3.32 per cent of arginine, and 8.10 per cent of lysine. It has been found that the silver compound of histidine can best be separated from that of arginine at pH 7.4 when the relative proportion of histidine to arginine is exceptionally high as is the case in hemoglobin. A careful examination of all the fractions produced in this analysis indicates that complete separations can be effected only by repeated precipitations and that the nitrogen content of the different fractions cannot be relied upon as a measure of the proportions of the bases in them. The best evidence of the actual composition of the histidine and arginine fractions is secured from the weights of their respective dinitronaphtholsulfonates and that of the lysine fraction from the weight of its picrate.

Evidence is presented that the precipitation of histidine by

Hopkins' reagent from a relatively pure solution of this base is practically complete.

The present results are in closest agreement with the assumption that the hemoglobin molecule, weighing 66,800, yields 33 molecules of histidine, 13 of arginine, and 37 of lysine.

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A COMPARISON OF THE TITRATION CURVES OF COAGULATED AND UNCOAGULATED EGG ALBUMIN.*

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A comparison of the titration curves of coagulated and uncoagulated egg albumin has been made in an effort to learn something of the process of coagulation of protein by heat. It is a well known fact that egg albumin and other proteins unite with acids in a medium of pH below 4.8 and with bases in a medium of pH above 4.8. The acid- or base-combining capacity varies with the increase in the hydrogen or hydroxyl ion concentration, but the combination is stoichiometric; the combining capacity at a given hydrogen ion concentration has been found to be a characteristic property. It is our purpose to show how heat-coagulated egg albumin compares with uncoagulated egg albumin in the ability to react with acids and bases. We employ titration values, combining capacities, and buffer values to test our hypothesis that heat coagulation causes a decrease in the number of acid- and base-combining groups; namely, the free amino and carboxyl groups of the protein molecule.

Loeb (1), Cohn (2), and Hoffman and Gortner (3) have reviewed experimental work on proteins which has thrown light on the properties of egg albumin. The divergent views of the colloid chemists who describe the combining of protein with acid or base by means of Freundlich's adsorption formula and those who regard these reactions as strict chemical combinations have led to a great deal of work, but not with entire agreement. Various methods have been employed to demonstrate the combining power

* This paper has been abstracted from a thesis presented by Miss Wilson to the Graduate School of the University of Texas in partial fulfillment of the requirements for the degree of Master of Arts.

of proteins, but accurate quantitative results were not obtained until the development of the electrometric method by Sørensen and others. The potentiometric method of measuring hydrogen and chloride ion concentrations in the presence of egg albumin was introduced by Bugarszky and Liebermann (4) in 1898.

Electrometric titrations of Sørensen (5), Loeb (6), and Hitchcock (7) showed that egg albumin is an ampholyte comparable to amino acids; the acid- or base-combining power increases with change in the hydrogen ion concentration on either side of the isoelectric point; the reaction of the protein with acid or base is stoichiometric, the combining power at a given pH being a characteristic property; and the salts formed by neutralization are highly ionized.

The first publication of titration curves is credited by Cohn (2) to D'Agostino and Quagliarello. Since then such curves have been widely used to describe the acidic and basic properties of proteins at various hydrogen ion concentrations. The results of titrations by Bugarszky and Liebermann (4), Loeb (6), Hitchcock (7), and Cohn and Berggren (quoted by Cohn (2)) agree well enough to be represented by means of one curve showing the amount of hydrochloric acid or sodium hydroxide combined with 1 gm. of crystalline egg albumin at varying hydrogen ion concentrations.

The process of the coagulation of egg albumin by heat has been studied by several investigators as a denaturation and subsequent flocculation (8-12). Chick and Martin (10), Lepeschkin (11), and others regarded denaturation as a hydrolysis. Lewis (13) considered denaturation to be a hydration of certain linkages—not peptide—in the protein molecule. He suggested that the affinity between the carboxyl and amino groups of the same molecule might be great enough to prevent reaction with the opposite groups of contingent molecules. Hydration of such linkages as the ethylene oxide, however, may introduce hydroxyl radicals between the carboxyl and amino groups and thus lessen their affinity for each other so that they are free to react with groups of adjacent molecules during flocculation. Robertson (14) concluded that coagulation is a process of dehydration of a heavily hydrated molecule.

EXPERIMENTAL.

The egg albumin used in this work was crystallized twice by the method of Hopkins and Pinkus (15). The solution of recrystallized egg albumin was now dialyzed in closed collodion bags to remove the ammonium sulfate. The distilled water in the dialyzing vessels was changed frequently. The dialysis was continued for a week or 10 days, until no sulfate was detected in the water of the outer vessel. Toluene was used as a preservative. After the completion of the dialysis, the albumin solution was filtered to remove the protein which had precipitated on the collodion

membrane, and the filtrate was kept in the refrigerator under toluene.

The nitrogen content of the stock solution was determined by the Kjeldahl method. Neither the sample nor the protein-free filtrate of the solution gave any evidence of the presence of ammonia, so it was assumed that the Kjeldahl nitrogen represented the protein nitrogen accurately. The factor 6.45 was used in calculating the total protein concentration ((5), p. 39). A determination of the heat-coagulated protein indicated that all of the protein was heat-coagulable.

A solution of this recrystallized egg albumin was brought to the isoelectric point by adding hydrochloric acid and the protein coagulated by heating for about 30 minutes in the autoclave at atmospheric pressure. This mixture was allowed to stand overnight; then it was filtered. The precipitated albumin was well washed with hot water and subsequently dried as much as possible by pressing between filter papers and washing with absolute alcohol. After it was thoroughly dried in an oven at 60°, it was pulverized and weighed for titrations.

Unfiltered, wet, coagulated albumin was also used. Volumes containing 1 gm. of protein were subjected to the heating process described above, but the filtration was omitted. This coagulated albumin was titrated in the mother liquor the day after it was coagulated. The hydrogen ion concentration was changed during coagulation so that a correction was introduced in this series to make the results comparable to those of other series.

The hydrogen ion concentrations of these protein-acid and protein-base solutions were determined with a quinhydrone electrode referred to a saturated calomel half-cell with a saturated potassium chloride bridge to eliminate contact potential. Electromotive force measurements were made with a Leeds and Northrup ion potentiometer. The potentiometric system was checked by determining the pH of standard (0.1 N) hydrochloric acid with each set of titrations.

The pH of the solution was calculated by the equation

$$\text{pH} = \frac{0.4538 - 0.00035(t - 18) - E}{0.0577 + 0.0002(t - 18)}$$

where t is the temperature and E is the measured E.M.F. (16).

The method used by Tague (17) was followed in the process of titration so that at a given pH the amount of protein and the total volume of solution were identical in all series. This precaution allowed a quantitative comparison of the volumes of acid or base necessary to bring 1 gm. samples of isoelectric albumin to a definite hydrogen ion concentration. Although the protein was coagulated in an isoelectric medium, the mixtures of dried albumin and water did not have a hydrogen ion concentration of pH 4.8. All other titrations, however, were begun with isoelectric solutions.

The standard hydrochloric acid was prepared by the Hulett and Bonner (18) constant boiling method and checked gravimetrically by precipitation with silver nitrate. The sodium hydroxide was standardized against this acid. Freshly boiled distilled water was used in all titrations.

Four sets of titrations were made as follows:

1. 1 gm. samples of dried coagulated albumin with water and varying amounts of 0.01 N acid or base were kept in the refrigerator and frequently shaken for about 17 days. At the end of this period and after the solutions had been allowed to come to room temperature, the solid quinhydrone was added. The protein-quinhydrone mixtures were well stirred to insure equilibrium before the final electrometric readings were taken.

2. To uncoagulated albumin solution, in volumes equivalent to 1 gm. of protein, water and 0.1 N acid or base were added from burettes in such amounts that the total volume and pH corresponded to those of the dried albumin series. The pH determinations were made as before.

3. Titrations of coagulated but unfiltered albumin were the same as the titrations of uncoagulated albumin; 0.1 N acid or base and water were added until the pH and total volumes corresponded to those titrations of dried coagulated egg albumin.

4. The titration of freshly boiled distilled water was necessary in order that we might estimate the amount of acid or base combined with the protein. Total volume precautions were heeded as before.

RESULTS AND DISCUSSION.

Tables I to V show the amount of acid or alkali necessary to bring the solution containing 1 gm. of egg albumin to a definite pH. The coagulated albumin curves deviate markedly from that of the uncoagulated albumin. The uncoagulated protein requires more of the titrating solution to change the hydrogen ion concentration of the mixture from the isoelectric point. The dried

TABLE I.
Titration of 1 Gm. of Dried Coagulated Egg Albumin with 0.01 N HCl.

Total volume of solution.	0.01 N HCl.	Temperature.	E.M.F.	pH	Total volume of solution.	0.01 N HCl.	Temperature.	E.M.F.	pH
Titration 1.					Titration 3.				
cc.	cc.	°C.	volt		cc.	cc.	°C.	volt	
79.0	0.0	26.0	0.1620	4.87	79.0	0.0	23.0	0.1650	4.89
84.0	5.0	25.5	0.2235	3.84	84.0	5.0	23.0	0.2270	3.83
89.0	10.0	25.5	0.2410	3.55	89.0	10.0	23.0	0.2465	3.50
97.0	18.0	25.5	0.2530	3.35	99.0	20.0	23.0	0.2520	3.41
109.0	30.0	25.5	0.2720	3.03	109.0	30.0	23.0	0.2690	3.12
119.0	40.0	25.5	0.2750	2.97	119.0	40.0	23.0	0.2770	2.98
129.0	50.0	25.5	0.2835	2.83	129.0	50.0	23.0	0.2830	2.88
Titration 2.					Titration 4.				
79.0	0.0	25.5	0.1380	5.29	79.0	0.0	24.0	0.1500	4.94
84.0	5.0	25.5	0.2215	3.88	84.0	5.0	24.0	0.2290	3.78
89.0	10.0	25.5	0.2250	3.82	89.0	10.0	24.5	0.2430	3.53
99.0	20.0	25.5	0.2460	3.46	99.0	20.0	24.5	0.2570	3.30
109.0	30.0	25.5	0.2650	3.14	109.0	30.0	24.0	0.2600	3.25
119.0	40.0	25.5	0.2750	2.97	119.0	40.0	24.0	0.2785	2.92
129.0	50.0	25.5	0.2830	2.84	129.0	50.0	24.0	0.2855	2.80

coagulated albumin requires less acid or base than the unfiltered coagulated albumin. For example, 66.5 cc. of 0.01 N HCl are required to change the unheated albumin solution from pH 4.85 to pH 3.0, while 46 cc. and 37 cc. are sufficient to produce the same pH change in the samples of coagulated unfiltered and dried coagulated albumin respectively. On the alkaline side, 37.8 cc. of 0.01 N NaOH decreased the hydrogen ion concentration of

originally isoelectric uncoagulated albumin to that of pH 7, but this same decrease was brought about by 11 cc. and 7.8 cc. of base in the titrations of coagulated unfiltered and dried coagulated albumins.

The change in the hydrogen ion concentration is due to only a fraction of the total acid or base; the remainder of the HCl or NaOH combines with the albumin to form albumin hydrochloride

TABLE II.

Titration of 1 Gm. of Dried Coagulated Egg Albumin with 0.01 N NaOH.

Total volume of solution.	0.01 N NaOH.	Temperature.	E.M.F.	pH	Total volume of solution.	0.01 N NaOH.	Temperature.	E.M.F.	pH
Titration 1.					Titration 3.				
cc.	cc.	°C.	volt		cc.	cc.	°C.	volt	
79.0	0.0	26.0	0.1620	4.87	79.0	0.0	23.0	0.1650	4.89
84.0	4.9	25.5	0.0250	7.20	84.0	4.9	23.0	0.0540	6.78
89.0	9.8	25.0	0.0320	7.06	89.0	9.8	23.0	0.0290	7.21
94.0	14.7	25.0	0.0080	7.49	94.0	14.7	23.0	0.0240	7.29
					99.0	19.5	23.0	0.0170	7.40
					104.0	24.4	23.0	0.0120	7.50
					114.0	34.3	23.0	-0.0060	7.80
Titration 2.					Titration 4.				
79.0	0.0	25.5	0.1380	5.29	79.0	0.0	24.0	0.1600	4.94
84.0	4.9	25.5	0.0510	6.76	84.0	4.9	24.0	0.0470	6.87
89.0	9.8	25.5	0.0340	7.05	89.0	9.8	24.5	0.026	7.20
94.0	14.7	25.5	0.0240	7.21	94.0	14.7	24.5	0.0200	7.30
99.0	19.5	25.5	0.0110	7.43	99.0	19.5	24.0	0.0170	7.38
104.0	24.4	25.5	-0.0080	7.76	104.0	24.4	24.5	0.0030	7.60
114.0	34.3	25.5	-0.0210	7.97	114.0	34.3	24.5	-0.0110	7.84

or sodium albuminate. The volume necessary to cause the pH change in the solution is shown by the water titration curve. The combination curves were constructed from the water curve and each of the titration curves. The difference, for example, in the volume of HCl required to bring 1 gm. of originally isoelectric uncoagulated albumin to pH 3.5 (with a total volume of 89 cc.) and the volume required to change the pH of water from 4.85 to 3.5 (with a total volume of 89 cc.) is the amount of HCl combined with

TABLE III.*

Titration of 1 Gm. of Unfiltered Coagulated Egg Albumin with 0.1 N HCl.

Total volume of solution.	HCl calculated as 0.01 N.	Temperature.	E.M.F.	pH
Titration 1.				
cc.	cc.	°C.	volt	
79.5	0.0	25.5	0.1610	4.90
79.8	3.0	25.5	0.1870	4.46
84.0	14.9	25.5	0.2235	3.84
89.0	19.9	25.5	0.2358	3.64
99.0	30.8	25.5	0.2550	3.31
109.0	39.8	25.5	0.2660	3.13
119.0	48.8	25.5	0.2750	2.97
129.0	57.7	26.0	0.2820	2.85
Titration 2.				
cc.	cc.	°C.	volt	
79.4	0.0	25.0	0.1598	4.93
79.8	3.0	25.0	0.1890	4.44
84.0	14.9	25.0	0.2260	3.81
89.0	17.9	25.0	0.2340	3.68
99.0	30.4	25.0	0.2560	3.30
109.0	36.8	25.0	0.2650	3.15
119.0	47.8	25.5	0.2750	2.97
129.0	55.7	25.5	0.2815	2.87
Titration 3.				
cc.	cc.	°C.	volt	
79.5	0.0	24.5	0.1739	4.71
79.8	3.0	24.5	0.1910	4.41
80.4	8.9	24.5	0.2130	4.04
83.5	10.9	24.5	0.2155	4.00
89.0	18.9	25.0	0.2350	3.66
99.0	29.9	25.0	0.2550	3.32
109.0	38.2	25.0	0.2660	3.15
119.0	47.8	25.0	0.2750	2.98
129.0	55.7	25.0	0.2820	2.86

* Some of our data have been omitted from this table.

1 gm. of the uncoagulated protein. Thus, at pH 3.5, 1 gm. of uncoagulated albumin is combined with 39 cc. of the 0.01 N HCl. This method of determining the combining capacity was used by

Loeb ((1) p. 55) in his titration of proteins and by Tague (17) in work with amino acids. The method takes into account neither the activity coefficient of the hydrogen ion in the protein solution

TABLE IV.

Titration of 1 Gm. of Unfiltered Coagulated Egg Albumin with 0.1 N NaOH.

Total volume of solution.	NaOH calculated as 0.01 N.	Temperature.	E.M.F.	pH
Titration 1.				
cc.	cc.	°C.	volt	
79.0	5.0	23.0	0.1090	5.84
79.1	6.2	23.0	0.0860	6.23
84.0	9.4	23.5	0.0570	6.71
89.0	12.3	23.5	0.0340	7.11
99.0	17.7	23.5	0.0130	7.46
109.0	22.6	24.0	0.0025	7.63
114.0	27.5	24.0	-0.0140	7.91
Titration 2.				
79.0	5.0	23.0	0.1040	5.93
81.5	7.0	23.0	0.0760	6.41
89.0	12.2	23.0	0.0360	7.07
99.0	18.7	23.5	0.0110	7.40
109.0	22.6	24.0	0.0037	7.61
114.0	29.4	24.0	-0.0140	7.91
Titration 3.				
79.0	5.0	25.0	0.0925	6.07
81.5	6.0	25.0	0.0755	6.36
84.0	7.9	25.0	0.0620	6.59
89.0	9.9	25.5	0.0368	7.00
99.0	17.7	25.5	0.0125	7.41
109.0	22.8	26.0	0.0030	7.55
114.0	28.5	26.0	-0.0125	7.82

nor the hydrolytic dissociation of protein hydrochloride, but it affords, nevertheless, an adequate basis for comparison of the combining capacities of the uncoagulated and coagulated proteins.

The curves in Fig. 1 show that the uncoagulated albumin com-

bines with more HCl or NaOH than the coagulated albumin. The dried coagulated albumin does not combine with as much of the acid or base as the unfiltered coagulated albumin, except in the region more alkaline than pH 7.3. We have no direct evidence of a shift in the isoelectric point due to coagulation.

In order to answer the question concerning the possibility of incomplete reaction between the insoluble coagulated egg albumin

TABLE V.*

Titration of 1 Gm. of Uncoagulated Egg Albumin with 0.1 N HCl and with 0.1 N NaOH.

Total volume of solution.	Acid or alkali calculated as 0.01 N.	Temperature.	E.M.F.	pH	Total volume of solution.	Acid or alkali calculated as 0.01 N.	Temperature.	E.M.F.	pH
Titration with HCl.					Titration with NaOH.				
cc.	cc.	°C.	volt		cc.	cc.	°C.	volt	
79.0	0.0	26.5	0.1603	4.89	79.0	0.0	25.5	0.1630	4.87
79.6	6.0	26.5	0.1750	4.64	79.6	5.9	25.5	0.1480	5.12
80.0	10.0	26.5	0.1840	4.49	80.0	9.8	25.5	0.1355	5.33
80.6	15.9	26.5	0.1970	4.27	80.4	13.7	25.5	0.1210	5.58
81.0	19.9	26.5	0.2040	4.15	80.8	17.6	25.5	0.1040	5.86
84.0	28.9	26.0	0.2205	3.89	81.5	19.5	25.5	0.0948	6.02
89.0	39.8	26.0	0.2385	3.58	84.0	26.4	25.5	0.0500	6.78
94.0	44.8	25.5	0.2449	3.48	89.0	29.3	26.0	0.0280	7.13
99.0	50.8	25.0	0.2543	3.33	94.0	31.3	26.0	0.0120	7.40
104.0	55.8	25.0	0.2610	3.22	99.0	32.2	26.0	0.0092	7.45
109.0	60.7	25.0	0.2660	3.14	104.0	33.2	26.0	0.0040	7.54
114.0	65.7	25.0	0.2710	3.05	109.0	33.9	26.0	0.0020	7.57
119.0	69.7	25.0	0.2750	2.98	114.0	38.1	26.0	-0.0160	7.87
129.0	79.7	25.0	0.2825	2.86					

* Some of our data have been omitted from this table.

and the acid or alkali of the solution, we have titrated by the same method one specimen each of acid and alkali metaprotein prepared by treating portions of recrystallized uncoagulated egg albumin with approximately 0.1 N HCl and 0.1 N NaOH respectively. The sample treated with HCl was allowed to stand overnight at room temperature and that treated with NaOH was left for 6 hours. The samples were then neutralized to the point of com-

plete precipitation of the metaprotein. The metaproteins were filtered, washed thoroughly, and dried. The alkali metaprotein

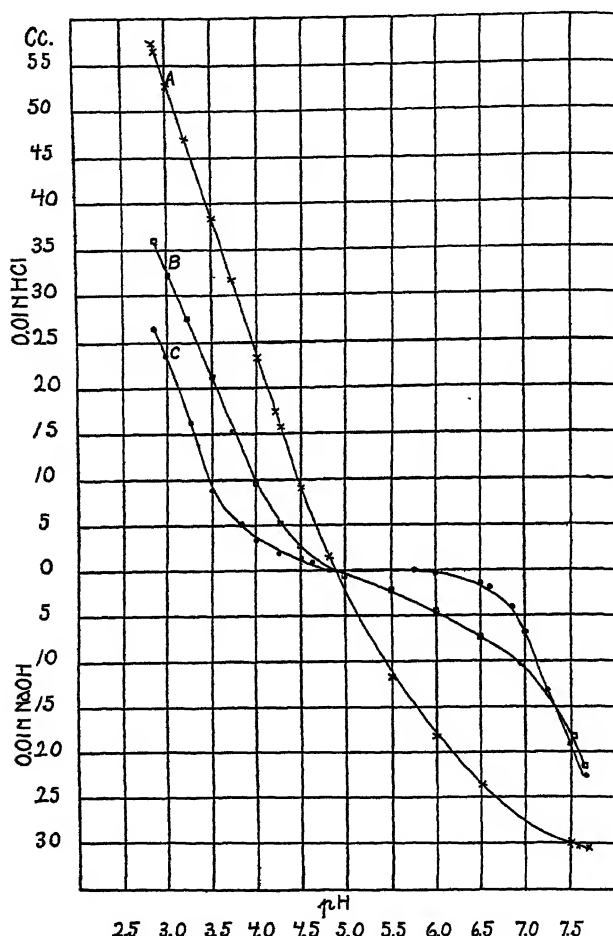


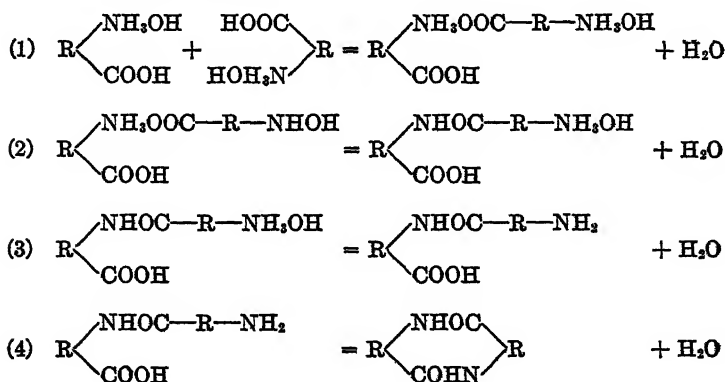
FIG. 1. The volume of 0.01 N HCl or NaOH combined with 1 gm. of egg albumin. A, uncoagulated egg albumin; B, coagulated unfiltered albumin; C, dried coagulated albumin.

was dried in a vacuum desiccator, while the acid metaprotein was dried in the refrigerator and finally in the desiccator. It was

found that the titration curves for these metaproteins were essentially parallel to the titration curve of uncoagulated egg albumin. This is especially true for considerable distances on either side of the isoelectric point, although the metaproteins appeared to be no more soluble than the coagulated protein in the presence of the same amounts of acid or alkali. We feel, therefore, that the flatness of the titration curves for coagulated egg albumin in the region of the isoelectric point is not due to a failure to establish equilibrium.

We have compared the titration curves of coagulated egg albumin after 2 days contact with the acid or alkali with the curves obtained after the protein had been in contact with the various solutions for 17 days, and we have found them essentially identical. This, we believe, shows that our curves are true titration curves of coagulated egg albumin.

The loss of combining capacity of the egg albumin during the process of coagulation is due to alteration of the degree of dissociation of the protein or to loss of free amino and carboxyl groups. The combination curves show that, within the pH range 2.7 to 7.7, at least, fewer acid and basic groups are functioning. It seems plausible that these groups have reacted during coagulation, probably giving up water. The process of dehydration described by Robertson (14) involves a decrease in the number of amino and carboxyl radicals. The protein molecule forms a loose combination with the water, with consequent dehydration on heating. The possibilities of reaction are:



Coagulation results, according to this theory, in the union of the carboxyl and amino groups to form anhydrides. There is, then, a decrease in the free acid and basic groups.

Two facts are pointed out by Lewis (13) which show the involvement of carboxyl and amino groups in the second step of coagulation: (1) coagulation is maximal in the isoelectric region, and (2) "on treatment of protein with formaldehyde the resulting denatured material even at a pH of isoelectric point exhibits greatly delayed flocculation owing to a portion of the amino groups being 'covered.'" A possibility of hydrolysis during denaturation is given by Lewis which involves some linkage other than the peptide, perhaps an ethylene oxide linkage. An introduction of hydroxyl groups between the amino and carboxyl groups of the same molecule lessens their affinity for each other so that they are free to react with the radicals of adjacent molecules, condensation and flocculation resulting.

If denaturation involves no change in the number of free amino and carboxyl groups (10) then flocculation must be more than a discharging of denatured material. There is, perhaps a removal of water due to a condensation of amino and carboxyl radicals rather than to a simple dehydration comparable to the removal of water of hydration of salts. On the other hand, if the flocculation of heat-denatured albumin is merely a physical process, then there must be some involvement of amino and carboxyl groups in denaturation which has not yet been revealed. Our results point to a chemical reaction during the process of coagulation which causes a decrease in the acid- and base-binding units of the protein molecule.

The combining power of dried coagulated albumin is less than that of the unfiltered coagulated albumin. This may be due to removal of water by drying. The process of drying may bring about a greater degree of condensation than heating alone. The dried coagulated albumin, under the conditions of these titrations, undoubtedly represents actual equilibrium to a greater degree than the unfiltered coagulated albumin. The difference in combination certainly shows a difference in the ability to ionize as an acid or base at a given pH, perhaps because more carboxyl and amino groups have reacted during the drying of the albumin.

The titration and combining curves show irregularities in the

alkaline region below pH 7. These may be due to inaccuracies of measurements with the quinhydrone electrode although this electrode is recommended for solutions as alkaline as pH 8 or 9. It is possible that some hydrolysis occurred in our more alkaline solutions. If this did happen, there would be liberated an increasing number of carboxyl groups which would neutralize some of the sodium hydroxide.

The egg albumin, which dissociates as a weak acid in a medium more alkaline than isoelectric point and as a weak base in a medium more acid, acts as a buffer. Van Slyke (19) adopts as a unit of

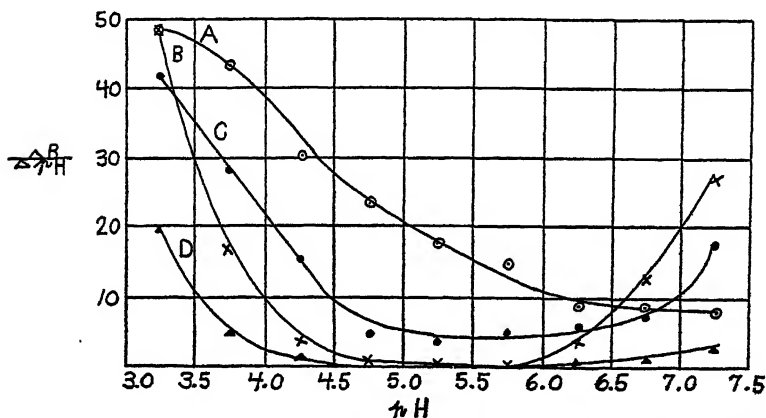


FIG. 2. Buffer values. A, uncoagulated albumin; B, dried coagulated albumin; C, unfiltered coagulated albumin; D, water.

measurement of buffer value the ratio $\frac{dB}{dpH}$, where dB is the increment of strong base and $-dB$ is the increment of strong acid added to a liter of the solution. The dpH is the increment in pH which results when the acid or base is added to the solution. We have constructed the buffer value curves with the ratio of measurable increments, $\frac{\Delta B}{\Delta pH}$, to compare the acid and basic properties of the three types of egg albumin. ΔB is the volume of 0.01 N acid or base necessary to change the pH 0.5. ΔpH , therefore, is 0.5. The difference in buffer values at a given pH depends upon the relative amounts of weak acid, or base, and the salt, and also

upon the degree of ionization of the free amino and carboxyl groups.

These buffer value curves (Fig. 2) show that coagulated albumin lies between uncoagulated albumin and water in its efficiency as a buffer. Since these titrations were not carried above pH 8, our buffer curves do not show the increase in buffer value near pH 9 which is revealed in the curves of titration of uncoagulated egg albumin by Loeb ((1), p. 68). The shape of the curves of both the dry and unfiltered coagulated albumin approaches that of the water curve, although the range of minimal buffer value is much less for the albumins than for water. It may be added that our water series was titrated from a pH of 4.8. It really amounts to the titration of a very dilute, unbuffered acid solution.

SUMMARY.

1. 1 gm. of uncoagulated egg albumin combines with more 0.01 N acid or base in the pH range 2.8 to 7.7 than 1 gm. of coagulated egg albumin.
2. 1 gm. of unfiltered coagulated egg albumin combines with more acid or base than 1 gm. of dried coagulated egg albumin.
3. The efficiency of heat-coagulated albumin as a buffer differs markedly from that of uncoagulated albumin.
4. It is suggested that heat coagulation involves a decrease in the number of free carboxyl and amino groups in the protein molecule. This decrease probably results from a condensation of opposite groups of adjacent molecules.
5. This condensation appears to be greater in the case of the dried coagulated egg albumin than in that of the unfiltered coagulated egg albumin.

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ON THE TIME OF ABSORPTION AND EXCRETION OF BORIC ACID IN MAN.

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In a previous article¹ it has been shown that boric acid passes through the living skin fairly rapidly. When the feet were dipped into a saturated aqueous boric acid solution up to the ankles, boric acid was found to be present in the urine in less than 5 minutes. The purpose of the present investigation is to ascertain more accurately just how long it takes for boric acid to appear in the urine after the feet are immersed as described.

Three men about 22 years of age and in perfect health served as subjects for the experiments. These men were 5 feet 9 inches, 5 feet 10 inches, and 6 feet in stature respectively. Their respective weights were 155, 163, and 188 pounds. Their feet were in excellent condition so far as could be determined by ordinary examination.

The experiments were conducted as follows: The bladder was emptied about an hour before the experiment was begun and neither food nor drink was taken while the experiment was in progress. Before the feet were immersed in boric acid a sample of urine was always taken. It was in each case found to be quite free from boric acid. Only one experiment was performed per day on any one person. Both feet were thoroughly washed with hot water and a little castile soap. They were then well rinsed with distilled water, wiped with a fresh, clean cloth, and at once immersed to a depth just a little above the ankles in a saturated aqueous solution of boric acid at about 45° in a porcelain-enameled dish. The feet rested but very lightly on the bottom of the dish, the subject sitting in a chair. The time of immersion and also

¹ Kahlenberg, L., *J. Biol. Chem.*, 1924-25, lxii, 149.

of each micturition was taken by means of a stop-watch. The samples of urine (about 25 cc. each time) were collected in clean glass vials and analyzed. About 5 minutes elapsed between the taking of the first and last samples, for previous work had shown that after this time boric acid is always present in estimable amounts.

In a preliminary series of ten experiments it was found that faint yet quite distinct tests for boric acid were obtained after the feet had been immersed for $2\frac{1}{2}$ minutes. Samples of urine taken before this time gave such extremely faint coloration to the turmeric paper (on account of the natural organic coloring matter in the urine) as to render the tests uncertain. These preliminary tests were made on the urine direct without the organic matter present being first destroyed, as described previously.² It was evident therefore that in order to get satisfactory tests on samples taken prior to 2 minutes, the organic matter in the samples of urine would have to be destroyed before the turmeric paper test was made. This was always done in the final series of experiments. The procedure consisted of making the sample of urine slightly alkaline by means of pure sodium bicarbonate, evaporating to dryness in a platinum dish, and igniting to destroy organic matter. The residue was then dissolved in a little water, acidified with a few drops of concentrated hydrochloric acid, and then tested with sensitive turmeric paper. The latter was moistened with the solution and dried on a large convex cover-glass on the water bath. The quantitative estimations were made colorimetrically by comparing the color of the turmeric paper with that of a color scale prepared with boric acid solutions of known strength.² In this way results that are quite satisfactory may be obtained. Indeed, by this method 1 part of boric acid in a million may still be detected and estimated. It is essential, however, that the turmeric paper used be fresh and of good, uniform quality.

The final results obtained are presented in Table I. It should be borne in mind that the object was not to find the total amount of boric acid secreted in a given time, but rather to determine the amount of boric acid in the urine at various intervals after

² Kahlenberg, L., *J. Biol. Chem.*, 1924-25, lxi, 152.

the immersion of the feet so as to ascertain how soon after the experiment is started it is possible to detect boric acid in the urine.

The first column of Table I gives the serial number of the samples, the second column the time elapsed after immersion of the feet, and the last column the observations as to boric acid content. When estimable quantities were present, the results are given in per cent. Each horizontal line of the table represents the average of ten samples. The first horizontal line gives the tests made before the feet were immersed, that is the blanks.

TABLE I.

Sample No.	Time.	Boric acid present.
1	0 sec.	None.
2	15 "	"
3	30 "	"
4	40 "	"
5	45 "	"
6	50 "	Faint test in 2 samples out of 10.
7	55 "	" " " 2 " " " 10.
8	60 "	" " " 7 " " " 10.
		<i>per cent</i>
9	1 min. 10 sec.	0.0005
10	1 " 15 "	0.00055
11	1 " 30 "	0.0006
12	1 " 45 "	0.0007
13	2 " 00 "	0.0008
14	2 " 30 "	0.00085
15	3 " 00 "	0.0009
16	5 " 00 "	0.001

With three exceptions, the experiments were conducted early in the morning; that is to say, on subjects who had been at rest for a considerable number of hours. Three series were, however, carried out late in the afternoon and immediately after the subjects had returned from a very brisk walk. Samples 6 and 7 in Table I give the results of two of these cases.

From the results in Table I it is evident that when the feet are immersed in saturated boric acid solution at 45°, boric acid passes into the urine in less than 1 minute; namely, 50 to 55 seconds.

This obviously represents the circulation time plus the time of absorption and secretion. The real time is probably somewhat less than that actually noted, for the observation depends on the sensitiveness of the test. Possibly if larger samples of urine had been taken earlier than 50 seconds and their residues tested, indication of the presence of boric acid might have been obtained even a little sooner. Physiologists have estimated the circulation time in man at about 20 to 30 seconds, the results, of course, being only approximate for in such experiments different paths are open to the blood in completing the circuit.

It is truly remarkable that the time required for the absorption of boric acid by the skin and its elimination by the kidneys is only about 50 seconds. So far as we are aware, the total time necessary for absorption by the skin, distribution by the circulation, and final excretion by the kidneys has never before been measured in the case of any substance.

THE IODINE CONTENT OF CAPE COD CRANBERRIES.*

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(Received for publication, July 6, 1928.)

The amount of iodine contained in cranberries grown in southeastern Massachusetts is of interest because the nearness of the sea affects the ground waters of the section. Normal chlorine in the spring-waters of the region ranges between 0.5 and 1.0 part in 100,000 parts of water, which is from 5 to 10 times the normal for the western part of the state (3). The vegetation of this region should consequently be richer in iodine than that of the interior of the country. Therefore, since Massachusetts produces the major portion of the nation's supply of cranberries, their iodine content possesses significance.

Three distinct lots of cranberries were analyzed: A mixed sample was prepared from several crates that were in storage awaiting shipment; a special lot was obtained from a bog on a narrow strip of land called Sandy Neck, projecting into Cape Cod Bay; while the third lot came from a bog in the center of Nantucket Island.¹

The cranberries were prepared for analysis in charges weighing 500 gm. each. The berries were cut in halves, then spread on trays, and placed in a drying oven where the temperature ranged between 60-75°. When dry, the material was ground in an iron mortar until all passed through a sieve with 1 mm. meshes. The dried charges varied in weight between 60 and 70 gm.

The analysis was based on the work of von Fellenberg (1).

* Published by permission of the Director of the Massachusetts Agricultural Experiment Station.

¹ The cranberries were secured through the kind cooperation of Mr. A. U. Chaney, of the American Cranberry Exchange, Mr. J. C. Makepeace of Wareham, and Mr. F. E. Smith of the Nantucket Cranberry Company.

The dry charge was mixed with about 25 gm. of finely powdered CaO and about 3 gm. of dry K_2CO_3 by grinding in a porcelain mortar until the mixture appeared to be homogeneous. Enough water was then mixed with it to slake the lime and form a crumbly mass. The charge was then spread in a layer in an iron bake-pan, $22 \times 12 \times 6$ cm., which would slide into the muffle of an electric furnace. The surface of the layer was sprinkled with enough CaO to cover it.

Heat was turned on gradually until the material was charred, care being used to avoid any glowing points on the surface of the mass. Heat was then increased and continued until the ash was white, which required a dull red heat of the muffle walls and several hours time.

The pan was removed from the furnace and when cool, the ash was washed into a porcelain mortar with water and ground to a thin paste with no gritty feel under the pestle.

The mass was next washed into a large flask with more water and heated to boiling for several minutes. After the solids had settled, the liquid was decanted into an evaporating dish. Boiling with water and decantation of the solution were repeated five or six times and finally the mass was thrown on a paper filter and washed twice. The total solution was evaporated to dryness or nearly so and the resultant residue repeatedly extracted with hot water. The second solution was evaporated to dryness, and from the original mass of ash and lime was obtained a small quantity of solids containing the iodine.

The succeeding steps followed the procedure of Jarvis, Clough, and Clark (2). The dry residue was repeatedly extracted with alcohol, which was then evaporated off. The residue was dissolved in water, acidified, oxidized with Cl , and titrated with $\text{N}/1000 \text{ Na}_2\text{S}_2\text{O}_3$ as in iodimetry.

Several charges from each lot of cranberries were analyzed, and the following results are the averages corrected for the iodine found in the reagents.

Cranberries from crates,	26	parts	iodine	per	billion.
" " Sandy Neck,	35	"	"	"	"
" " Nantucket,	27	"	"	"	"

That the iodine was highest in the fruit from Sandy Neck is consistent with its closeness to the sea; but the total crop is better represented by the other figures.

Iodine has seldom been reported in analyses of plants. Some results published by von Fellenberg (1) are given here for comparison with the cranberries.

Apples without cores contained 1 to 6 parts of iodine per billion; orange pulp, 16 parts; potatoes, 11 to 18 parts; cabbage, 6 to 20 parts; lettuce, 6 to 18 parts. The ranges in figures represent the differences found between European districts, which were respectively unfavorable and favorable for the intake of iodine by plants.

By comparison with these analyses, cranberries from southeastern Massachusetts have a relatively high content of iodine.

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THE AVAILABILITY OF DISULFIDE ACIDS AS SUPPLEMENTING AGENTS IN DIETS DEFICIENT IN CYSTINE.

II. α -DIHYDROXY- β -DITHIODIPROPIONIC ACID.

By BEULAH D. WESTERMAN* AND WILLIAM C. ROSE.

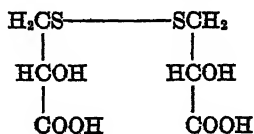
(From the Laboratory of Physiological Chemistry, University of Illinois, Urbana.)

(Received for publication, July 16, 1928.)

Having shown in a former paper (Westerman and Rose, 1927) that dithiodiglycollic acid and β -dithiodipropionic acid are incapable of replacing cystine of the diet for purposes of growth, we next undertook experiments of a similar sort with α -dihydroxy- β -dithiodipropionic acid. The latter is an interesting compound from the biochemical point of view because it bears the same structural relationship to cystine that *dl*- β -4-imidazole lactic acid does to histidine. Investigations in this laboratory (Cox and Rose, 1926) and elsewhere (Harrow and Sherwin, 1926) have shown that *dl*- β -4-imidazole lactic acid can induce growth in animals receiving diets adequate in every respect except as regards the histidine content. It seemed important, therefore, to determine whether a similar replacement of cystine by the corresponding hydroxy acid can be accomplished by the organism.

EXPERIMENTAL.

After numerous unsuccessful attempts to synthesize α -dihydroxy- β -dithiodipropionic acid (I) from simpler compounds, we



I.

* The experimental data in this paper are taken from a thesis submitted by Beulah D. Westerman in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Physiological Chemistry in the Graduate School of the University of Illinois.

finally resorted to the procedure described by Neuberg and Ascher (1907) for the preparation of the barium salt. The principle involved is very simple, and consists in the treatment of cystine in sulfuric acid solution with barium nitrite, rendering the solution alkaline with barium hydroxide, removing the excess barium with carbon dioxide, and precipitating the barium α -dihydroxy- β -dithiodipropionate by the addition of alcohol. No difficulty is encountered in securing satisfactory yields of the barium salt, but the separation of the free acid in pure condition is complicated by the fact that the product is rather unstable toward heat, and is recovered as a syrup which it is difficult to purify or dry. Neuberg and Ascher state that the free acid may be obtained by decomposing the barium salt with sulfuric acid, but there is nothing in the paper to indicate that the authors attempted to recover the product in *dry form*. The approximate specific rotation of the free acid reported by them evidently was calculated from the amount of barium salt decomposed to give the acid solution.

We have found it advantageous not to separate the barium salt, but to extract the free acid with ether immediately after the completion of the nitrite reaction. The process in full is as follows: 16 gm. of cystine are dissolved in 270 cc. of normal sulfuric acid by warming on the steam cone. The solution is cooled to 0°, and while being agitated with a mechanical stirrer, is treated with an aqueous solution of 27 gm. of barium nitrite. The latter is added drop by drop to avoid the evolution of red fumes. When the addition is completed, the whole is allowed to stand in a freezing mixture for a short time, and at room temperature for several hours. It is then warmed until the evolution of gas ceases. After the mixture is cooled, the precipitate of barium sulfate is filtered off. The filtrate is concentrated *in vacuo* to a volume of approximately 150 cc., and extracted several times with dry (alcohol-free) ether. After removal of the ether by evaporation, the α -dihydroxy- β -dithiodipropionic acid remains as a pale yellow, sticky residue which, after desiccation for several weeks *in vacuo* over sulfuric acid, may be reduced to a powder. Various pure and mixed solvents were employed in an attempt to crystallize the material, and thus remove the slight color, but none proved successful.

An aqueous solution of our solid material yields a white precipitate with mercuric chloride, and a green insoluble salt on being warmed with cupric acetate. The addition of silver nitrate produces a white flocculent precipitate. These reactions are in accord with those reported by Neuberg and Ascher for the barium salt. The acid was devoid of nitrogen, and failed to respond to the cysteine and cystine tests of Sullivan (1926).

Elementary analyses gave the following results as compared with the theory:

	Calculated. per cent	Found. per cent
Carbon.....	29.74	30.07
Hydrogen.....	4.16	4.28
Sulfur.....	26.47	25.70
Oxygen.....	39.63	39.95 (By difference.)

As will be seen, the sulfur content is approximately 0.8 per cent low, while the carbon, hydrogen, and oxygen are somewhat high. These facts suggest that the compound was partially desulfurized in process of preparation. In this connection should be mentioned the fact that numerous attempts to racemize portions of the compound by refluxing in organic solvents invariably yielded products which, though inactive toward polarized light, were 2 to 3 per cent low in sulfur. Evidently, the latter is removed quite readily.

Despite the fact that the compound in the syrupy state retains moisture with great tenacity, when once thoroughly dried it redissolves in water very slowly. Determinations of the specific rotation were made in acetone or ethyl acetate. In acetone, 2 per cent solutions of two different preparations showed at 23° specific rotations of -10.4° and -11.3° respectively. At the same temperature and concentration in ethyl acetate the specific rotation amounted to -15.6°. These values are approximate only since the slight yellow color of the solutions prevented sharp polariscopic readings. Neuberg and Ascher report for their preparation in aqueous solution an approximate specific rotation of -10.6°. Our product did not give a sharp melting point as obviously it was not chemically pure. Several preparations were tested and behaved similarly. Each softened at about 80° and melted between 85° and 90°.

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In order further to identify the acid a derivative was prepared by shaking a solution in dilute sodium carbonate with the calculated amount of *p*-nitrobenzoyl chloride. On acidification of the filtered mixture the di-*p*-nitrobenzoate of α -dihydroxy- β -dithiodipropionic acid precipitated. Sulfur determinations showed that the derivative contained 11.84 per cent as compared with the theoretical value of 11.87 per cent. This finding and the results of the elementary analyses indicate that about 97 per cent of our dry material consisted of the desired product.

The feeding experiments were conducted in a manner analogous to those described in a former paper upon disulfide acids (Westerman and Rose, 1927). Litter A (Chart I) received during the first 29 days the modified Sherman-Merrill (1925) ration containing 16.66 per cent of whole milk powder. The proportion of milk powder was then reduced to 12.0 per cent with a corresponding increase in corn-starch. This change was made in order to reduce somewhat the cystine content of the basal diet. Litter B (Chart II) received the lower proportion of milk powder throughout. The diet as finally modified had the following composition:

	<i>per cent</i>
Whole milk powder.....	12.00
Corn-starch.....	86.33
Sodium chloride.....	1.67

Vitamins were supplied to each animal daily in the form of 200 mg. of commercial yeast and 6 drops of cod liver oil. Both the yeast and the oil were administered apart from the rest of the diet. This ration, when supplemented with 0.2 per cent of cystine, supports steady growth at a rate about two-thirds the normal maximum. When cystine or α -dihydroxy- β -dithiodipropionic acid was included in the food, the substance in question replaced an equivalent amount of starch. The quantities of the supplementing sulfur compounds employed were:

	<i>per cent</i>
Cystine.....	0.2
α -Dihydroxy- β -dithiodipropionic acid.....	0.2 or 0.4

The results of the experiments are summarized in the charts. Rats 846, 847, and 848 (Chart I) received the disulfide acid at a

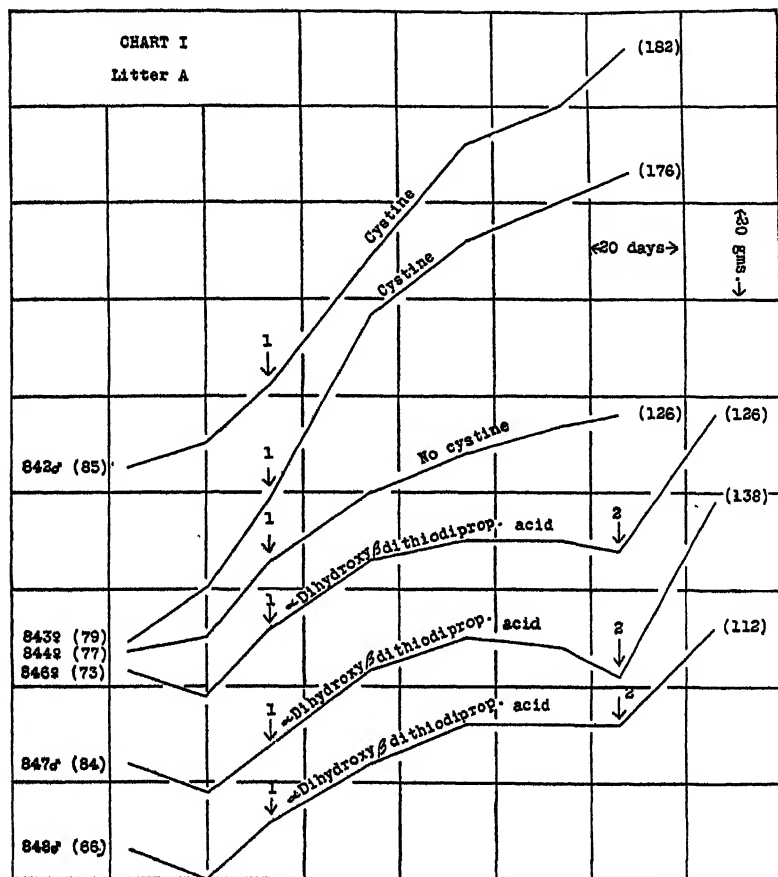


CHART I. The number and sex of each rat is shown at the extreme left of the chart. The figures in parentheses signify the initial and final weights of the animals. For the entire period of the experiment Rats 842 and 843 were given cystine. Rat 844 received neither cystine nor the disulfide acid. To Rats 846, 847, and 848, 0.2 per cent of the disulfide acid was administered continually. During the first 29 days the basal diet of all the animals contained 16.66 per cent of whole milk powder; thereafter the latter was reduced to a 12.0 per cent level. This change is indicated on the curves by the figure 1. At the points denoted by figure 2, cystine *in addition to the disulfide acid* was incorporated in the food.

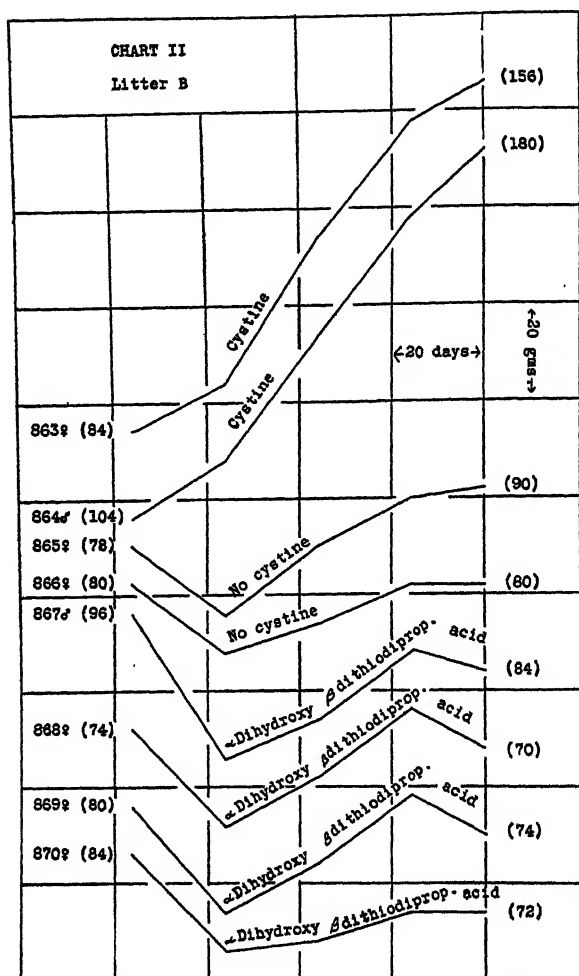


CHART II. Each animal received throughout the experiment a basal diet containing 12.0 per cent of whole milk powder plus the supplementing agent denoted on the individual curves. The diet administered to Rats 867, 868, 869, and 870 contained 0.4 per cent of the disulfide acid, or approximately twice the equivalent of the cystine given to the control animals.

0.2 per cent level. Rats 867, 868, 869, and 870 (Chart II) were given 0.4 per cent, or approximately twice the equivalent of the cystine administered to the control animals. The curves indicate that α -dihydroxy- β -dithiodipropionic acid, when fed at either level, is

TABLE I.
Food Consumption of Experimental Animals.

Litter.	Rat No.	Diet.	Average daily food consumption.
			gm.
A	842	Basal* + cystine.	9.6
		"† + "	11.6
	843	"* + "	9.9
		"† + "	11.4
	844	"*	9.5
		"†	10.7
	846	"* + disulfide acid.	7.5
		"† + " "	7.9
		"† + " " + cystine.	9.6
	847	"* + " " "	8.0
		"† + " " "	8.9
		"† + " " + cystine.	9.7
B	848	"* + " " "	6.6
		"† + " " "	7.7
		"† + " " + cystine.	9.3
	863	Basal† + cystine.	11.6
	864	"† + "	12.6
	865	"†	8.7
	866	"†	7.6
	867	"† + disulfide acid.	7.0
	868	"† + " "	6.8
	869	"† + " "	6.6
	870	"† + " "	7.4

* Basal diet contained 16.66 per cent of whole milk powder.

† Basal diet contained 12.0 per cent of whole milk powder.

incapable of serving in place of cystine. Indeed, the rats which received the synthetic compound made less satisfactory gains and consumed less food than did their litter mates upon the basal diet without added cystine (*cf.* Table I). This observation is in accord with results of previous experiments involving the use of

dithiodiglycollic and β -dithiodipropionic acids (*cf.* Westerman and Rose, 1927). Inasmuch as the basal diet without added cystine and the diet containing the disulfide acid both proved to be inadequate for growth, the differences in food intake under the two conditions may have been due to the taste of the synthetic compound. On the other hand, the addition of 0.2 per cent of cystine to the food containing the disulfide acid was followed immediately by the rapid growth of the animals accompanied by an increased food intake (see Chart I and Table I; Rats 846, 847, and 848). Evidently the synthetic compound prevented neither growth nor the consumption of sufficient food when the cystine deficiency was terminated. It should be pointed out also that despite differences in food intake, the rats upon the higher level of disulfide acid (Chart II) actually ingested amounts of this material which were more than equivalent to the quantities of cystine consumed by their litter mates. Thus the unsatisfactory growth of the former is not to be attributed to an insufficient intake of the synthetic compound.

The inability of α -dihydroxy- β -dithiodipropionic acid to replace cystine in metabolism is an interesting contrast to the ready substitution of *DL*- β -4-imidazole lactic acid for histidine. McGinty, Lewis, and Marvel (1924-25) have shown that α -hydroxy- ϵ -amino-caproic acid is incapable of serving for lysine; and more recently Jackson (1927) has reported that *L*- β -3-indole lactic acid does not induce growth when added to a diet deficient in tryptophane. Apparently, histidine is the only one of the four amino acids known to be essential which can be replaced for growth purposes by the corresponding hydroxy acid. This difference may be due to the total inability of the organism to synthesize lysine, tryptophane, and cystine from the corresponding hydroxy acids; or the syntheses may occur but at rates too slow to meet the demands of growth. We have experiments planned which we hope will further elucidate this point.

SUMMARY.

Synthetic α -dihydroxy- β -dithiodipropionic acid is incapable of replacing cystine of the diet for purposes of growth. Either the amino acid cannot be formed from the corresponding hydroxy acid

under the conditions of our experiments, or its synthesis is not sufficiently rapid to meet the growth requirements of the organism.

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THE OXIDATION OF DISULFIDE ACIDS IN THE ANIMAL ORGANISM.

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Inasmuch as dithiodiglycolic, β -dithiodipropionic, and α -dihydroxy- β -dithiodipropionic acids cannot serve in place of cystine for the growth of rats (Westerman and Rose, 1927, 1928), it seemed of interest to determine whether the compounds in question can undergo oxidation in the animal organism. As far as we are aware, the fate of the above disulfide acids has not been investigated hitherto. Indeed, the literature affords comparatively little information concerning just what types of sulfur compounds may be oxidized in the body. Wolf and Osterberg (1912) and others have shown that the sulfur of cystine is almost completely transformed to sulfuric acid, and reappears as sulfates in the urine. Likewise cysteine is readily burned (Dakin, 1913; Sherwin, Shiple, and Rose, 1927), as are other aliphatic mercapto compounds (Hill and Lewis, 1924). On the contrary, organic sulfides (Hill and Lewis, 1924), sulfonic acids such as taurine, cysteic acid, and isethionic acid (Schmidt and Clark, 1922), and certain other sulfur compounds, notably thiopyrimidines (Steudel, 1903) and thiourea (Masuda, 1910), are oxidized with difficulty, if at all. According to Lewis (1924), "it appears that of the various types of sulfur linkages investigated, only mercapto groups or such others as may be hydrolyzed (thiourethane) or reduced (cystine) to form this group are readily oxidized in the organism." Since our three acids are capable of yielding mercapto

* The experimental data in this paper are taken from a thesis submitted by Beulah D. Westerman in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Physiological Chemistry in the Graduate School of the University of Illinois.

groups on being reduced, we anticipated that they would undergo oxidation with ease.

EXPERIMENTAL.

Rabbits were employed as the experimental animals. As in the procedure of Hill and Lewis (1924), the daily diet consisted

TABLE I.
Oxidation of Dithiodiglycollic Acid.

Day.	Urine.					Remarks.
	Total N.	Total S.	Total SO ₄ -S.	Inorganic SO ₄ -S.	Unoxidized S.	
Rabbit 1, male, 2.6 kilos.						
	gm.	mg.	mg.	mg.	mg.	
1	0.93	47.8	27.2	21.3	20.6	
2	0.94	51.2	35.7	26.5	15.5	
3	0.94	49.4	34.9	24.0	14.5	
4	0.92	133.4	96.7	85.1	36.7	0.25 gm. acid given <i>per os</i> as sodium salt.
5	0.90	129.7	93.3	81.6	36.4	" "
6	0.90	47.6	36.4	29.1	11.2	
7	0.92	59.3	41.0	36.9	18.3	
Rabbit 2, male, 2.4 kilos.						
1	0.86	35.3	23.4	8.9	11.9	
2	0.90	37.5	23.5	9.6	14.0	
3	0.80	34.3	20.4	11.8	13.8	
4	0.74	112.6	44.3	35.2	68.3	0.25 gm. acid given <i>subcutaneously</i> as sodium salt.
5	0.81	117.0	47.1	37.6	69.9	" "
6	0.86	41.9	24.8	16.5	17.1	
7	0.89	52.0	34.0	24.9	18.0	

of 150 cc. of milk, 10 gm. of sucrose, 10 gm. of hay, and water *ad libitum*. The animals consumed the diet readily and completely, and in all cases maintained their body weights very constantly. The basal ration was administered for several days preceding the beginning of each experiment in order to establish nitrogen equilibrium. The urines were collected at 24 hour inter-

vals by gentle pressure upon the abdomen. Total sulfur was determined by Denis' (1910) modification of Benedict's (1909) procedure. Total and inorganic sulfates were estimated according to the Folin (1905-06) methods. Total nitrogen determinations

TABLE II.
Oxidation of β -Dithiodipropionic Acid.

Day.	Urine.					Remarks.
	Total N.	Total S.	Total SO ₄ -S.	Inorganic SO ₄ -S.	Unoxidized S.	
Rabbit 3, male, 2.3 kilos.						
	gm.	mg.	mg.	mg.	mg.	
1	0.82	41.9	28.5	17.1	12.4	
2	0.76	43.4	30.0	19.5	13.4	
3	0.76	47.1	26.0	20.8	21.1	
4	0.75	49.4	34.3	25.5	15.1	
5	0.81	191.5	118.0	102.5	73.5	0.5 gm. acid given <i>per os</i> as sodium salt.
6	0.84	139.5	84.7	74.1	54.8	" "
7	0.55	90.8	49.8	46.9	40.9	
8	0.82	53.1	32.3	28.1	10.8	
9	0.91	52.9	34.0	26.8	18.8	
Rabbit 4, male, 2.3 kilos.						
1	1.34	89.9	62.2	49.0	27.7	
2	0.94	61.5	40.2	32.8	21.3	
3	1.01	62.5	44.3	33.8	18.2	
4	1.06	210.4	117.0	110.5	93.4	0.5 gm. acid given <i>per os</i> as sodium salt.
5	0.98	209.2	129.9	124.2	78.3	" "
6	0.95	65.7	46.5	32.9	19.2	
7	0.96	76.5	52.3	36.4	24.2	
8	1.00	76.0	53.9	43.5	22.1	

were made daily in order to exclude the possibility of an exaggerated protein catabolism which might contribute an increased sulfur excretion. For this purpose the Scales and Harrison (1920) modification of the Kjeldahl procedure was employed.

The results of the individual experiments are presented in

Tables I to III. In Table IV the data are summarized, and calculations are made of the percentage of oxidation of the three disulfide acids. As will be observed, each of the compounds led to an increased output of sulfate sulfur. Calculations show that following the oral administration of 0.25 gm. of dithiodiglycollic acid on 2 succeeding days, approximately 78 per cent of the sulfur

TABLE III.
Oxidation of α -Dihydroxy- β -Dithiodipropionic Acid.

Day.	Urine.					Remarks.
	Total N.	Total S.	Total SO ₄ -S.	Inorganic SO ₄ -S.	Unoxidized S.	
Rabbit 5, male, 1.8 kilos.						
1	0.74	43.7	31.0	19.5	12.7	0.25 gm. acid given <i>per os</i> as sodium salt. " "
2	0.71	33.6	21.0	14.4	12.6	
3	0.77	39.6	24.5	13.3	15.1	
4	0.73	73.9	50.8	37.7	23.1	
5	0.73	76.5	43.7	34.6	32.8	
6	0.72	49.4	28.2	20.8	21.2	
7	0.81	60.8	35.0	24.5	25.8	
Rabbit 6, male, 1.5 kilos.						
1	0.68	25.4	13.3	8.2	12.1	0.25 gm. acid given <i>subcutaneously</i> as sodium salt. " "
2	0.71	23.5	16.5	11.0	7.0	
3	0.68	20.6	15.8	6.6	4.8	
4	0.71	58.5	22.2	12.5	36.3	
5	0.76	84.5	29.3	27.8	55.2	
6	0.76	34.5	20.4	16.9	14.1	
7	0.73	32.9	15.4	8.2	17.5	

was oxidized. Subcutaneous administration resulted in the oxidation of about 34 per cent. Perhaps the higher figure under the former condition may have been due to a less rapid absorption with greater opportunity for oxidation, or to the aid of bacterial action in the intestine.

Two experiments involving the oral administration on 2 suc-

ceeding days of 0.5 gm. doses of β -dithiodipropionic acid show that about 56 and 51 per cent respectively of the sulfur reappeared in the urines as sulfates. Evidently this compound, like dithiodiglycollic acid, is quite readily burned in the organism.

The results of the two experiments with α -dihydroxy- β -dithiodipropionic acid show that following the oral and subcutaneous administration of this disulfide approximately 42 and 20 per cent respectively were oxidized as measured by the increase in output of sulfates. These are somewhat lower values than were secured

TABLE IV.
Percentage Oxidation of Disulfide Acids.

Compound.	Amount given.	S content of acid given.	Expected excretion of total $\text{SO}_4\text{-S}$.*	Observed excretion of total $\text{SO}_4\text{-S}$.	Extra $\text{SO}_4\text{-S}$.	Administered S oxidized.
	gm.	mg.	mg.	mg.	mg.	per cent
Dithiodiglycollic acid (<i>per os</i>)....	0.5	176.0	228.2	365.2	137.0	77.8
“ “ (subcutaneously).....	0.5	176.0	157.0	217.5	60.5	34.3
β -Dithiodipropionic acid (<i>per os</i>)..	1.0	305.0	267.3	437.6	170.3	55.8
“ “ “ “	1.0	305.0	391.2	546.3	155.1	50.9
α -Dihydroxy- β -dithiodipropionic acid (<i>per os</i>).....	0.5	132.4	178.5	234.2	55.7	42.1
α -Dihydroxy- β -dithiodipropionic acid (subcutaneously).....	0.5	132.4	106.4	132.9	26.5	20.0

* Calculated from the output of total $\text{SO}_4\text{-S}$ during the days preceding the administration of the disulfide acid.

with dithiodiglycollic acid and β -dithiodipropionic acid. On the other hand, the total recovery of administered sulfur was less with α -dihydroxy- β -dithiodipropionic acid than with either of the other two compounds. Whether this indicates that part of the hydroxy acid was utilized by the organism is not evident. The fact in which we are particularly interested at the present time is that all three of our synthetic disulfide acids undergo appreciable oxidation in the animal body despite their inability to replace cystine for purposes of growth.

SUMMARY.

Experiments have shown that although dithiodiglycollic acid, β -dithiodipropionic acid, and α -dihydroxy- β -dithiodipropionic acid cannot replace cystine for the growth of rats, the compounds in question readily undergo oxidation when administered orally or subcutaneously to rabbits.

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SOME RECENT DETERMINATIONS OF ASPARTIC AND GLUTAMIC ACIDS IN VARIOUS PROTEINS.*

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The importance of having as accurate data as possible regarding the quantities of the different amino acids yielded by proteins on hydrolysis is so generally recognized as to need here no special emphasis. Our knowledge of the amino acid content, however, even of the best known proteins is very incomplete and inadequate, with the exception of a few cases in which recently developed methods of analyses have been applied.

In an attempt to locate the sources of loss when analyzing the products of protein hydrolysis by the ester method of Emil Fischer, Osborne and Jones (1) were able to recover only 66.2 per cent of the amino acids originally present in an artificial mixture of pure amino acids after the mixture had been subjected to the same procedure as employed in the hydrolysis and analysis of proteins by the ester method. Only 42.5 per cent of the aspartic acid was recovered, and 69.4 per cent of the glutamic acid. Experience with recent methods for the determination of these amino acids has shown that the actual percentages of aspartic and glutamic acids in many proteins are far higher than those recorded for them in the literature even after corrections have been made on the basis of the percentage recoveries recorded by Osborne and Jones.

Most of these reported percentages were obtained by the ester method, and the amino acids were isolated from the high boiling fractions of the distilled esters, and from the distillation residues.

* A preliminary report of this work was presented at the twenty-first annual meeting of the American Society of Biological Chemists held in Rochester, April 14-16, 1927.

The ester method involved severe losses due largely to incomplete esterification, decomposition of the esters during their preparation and distillation, and the formation of mixtures containing amino acids which could not be separated. A loss of glutamic acid unappreciated at that time frequently occurred as a result of the conversion of this amino acid into pyrrolidonecarboxylic acid yielding sirupy products that could not be identified, and which were usually discarded. These sirups furthermore made it very difficult to isolate the aspartic acid that was usually present in these fractions, thus accounting for another source of loss of that amino acid.

In many cases, however, the greater part of the glutamic acid was isolated directly as the hydrochloride before esterification, by saturation of the protein hydrolysate with hydrochloric acid gas. The remaining portion of the glutamic acid, which escaped separation as the hydrochloride, was subsequently incompletely recovered from the high boiling fractions of the esters. Under these circumstances only a small portion of the glutamic acid present in the protein was subjected to the losses involved in the analysis by the Fischer ester method. All the aspartic acid present, on the other hand, was subjected to these losses, since it was obtained entirely from the distilled esters. For this reason, the percentages given in the literature for glutamic acid in most proteins represent more nearly the quantities actually present than those given for aspartic acid. This accounts for the fact that the differences between the old figures and those obtained by more recent methods of analysis are much greater for aspartic acid than for glutamic acid.

In 1914, Foreman (2) showed that the dicarboxylic amino acids could be practically quantitatively separated as a group from most of the other products in protein hydrolysates by precipitation of their calcium salts with alcohol. The same method in which the barium salts were used (3, 4) has been later successfully applied. In the three or four cases where this method has already been applied much higher percentages of aspartic and glutamic acids have been isolated than were formerly obtained by the ester method.

The work described in this paper was undertaken with the view of obtaining by the use of the more recently developed methods of

analysis more accurate information regarding the percentages of these amino acids in several important proteins. To this end attention has been given particularly to the conditions and sequence of the various analytical procedures involved that would give the most satisfactory results.

Some thirteen proteins, representative of important food proteins and also of different classes of proteins, have been analyzed. In every case the percentages found for aspartic acid in the different proteins analyzed are higher than those previously recorded and obtained by the ester method. Many of the figures are several times higher, that for lactalbumin exceeding the old figure by 900 per cent. It is of interest to note that the 10 per cent of aspartic acid isolated from edestin is the highest ever recorded for that amino acid in any protein. The increases over the old figures for glutamic acid, although quite significant, are not so striking as those for aspartic acid.

Although the procedure and sequence of operations described in the analysis of edestin usually gave the best results, it was found, however, that they had to be varied more or less with different proteins. In the case of proteins containing high percentages of glutamic acid it is preferable to remove as much as possible of this amino acid as the hydrochloride before precipitation of the calcium or barium salts.

It was found more advantageous to precipitate the dicarboxylic amino acids as their barium salts than as the calcium salts. With the barium salts the precipitation is more complete, excess of the reagent is more easily removed, and pure barium hydroxide is more available than pure lime.

The barium salts of the dicarboxylic amino acids are practically quantitatively precipitated by pouring their aqueous solution into 5 volumes of 95 per cent alcohol. Varying amounts of other amino acids may also be precipitated, notably tyrosine, glycine, and diamino acids. When the precipitated barium salts are dissolved in water and reprecipitated with 2 volumes of alcohol, the salts of the dicarboxylic amino acids separate almost completely, while most of the other material remains in solution. In some cases even a third precipitation may be advisable.

Solutions of the amino acids were concentrated by distillation at reduced pressure at temperatures not above 50°. The use of

higher temperatures was avoided as far as possible so as to reduce to a minimum the conversion of glutamic acid to pyrrolidone-carboxylic acid. Even with such precautions the conversion occurred in some cases to a considerable extent.

The percentage of water of crystallization in many of the preparations of air-dried copper aspartate varied from the $4\frac{1}{2}$ mole-

TABLE I.
Aspartic and Glutamic Acids in Various Proteins.

Protein.	Aspartic acid.		Glutamic acid.	
	Determined by recent methods.	Determined by ester method.	Determined by recent methods.	Determined by ester method.
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Glycinin (soy bean).....	9.4	3.9	18.5	19.5
Edestin (hemp seed).....	10.2	4.5	19.2	18.7
Ovalbumin (hen's eggs).....	6.2	2.2	13.3	9.1
Lactalbumin*.....	9.3	1.0	12.9	10.1
Stizolobin (velvet bean)*.....	9.2	5.7		14.6
Fish muscle (halibut).....	8.0	2.7	13.7	10.1
Ox muscle.....	5.9	4.5	13.4	15.5
Shrimp muscle*.....	7.0		15.0	
Gliadin (wheat).....	0.8	0.6	43.0	43.7
Glutenin ".....	2.0	0.9	25.7	23.4
Arachin (peanut).....	5.6	3.8	19.5	16.7
Kafrin (kafir)*.....	2.3	0.6	21.2	
Locust bark albumin*.....	7.7		4.5	
Gelatin†.....	3.4	1.2	5.8	1.7
Zein†.....	1.8	1.7	31.3	26.2
Casein†.....	4.1	1.4	21.0	15.6

* Although the figures for these proteins have appeared in previous publications from this laboratory, they are included in this table because they were determined for the greater part by the newer methods.

† The figures for gelatin, zein, and casein given in the columns under "Determined by recent methods" are those of Dakin (3, 10, 11).

cules usually given in the literature for this salt. All analyses of copper aspartate, therefore, were made on the anhydrous salt, prepared by heating the air-dried product *in vacuo* (about 20 mm.) over phosphorus pentoxide for from 2 to 3 hours at 125°.

The proteins analyzed were especially prepared and purified. The percentages of aspartic and glutamic acids reported represent quantities isolated, the purity of which was definitely established.

The results of these analyses are summarized in Table I. There are also given for comparison the highest, and what seems the most reliable percentages that had been previously obtained for the same proteins by the Emil Fischer method. Figures for a few other proteins which have been already reported in previous publications from this laboratory (lactalbumin (5), shrimp muscle (6), kafirin (7), locust bark albumin (8), and stizolobin, the globulin of the Chinese velvet bean (9)), are here brought together because the percentages given were obtained by practically the same methods as those just described. The figures more recently obtained by Dakin for gelatin (3), zein (10), and casein (11) by newly developed methods are also incorporated in Table I, both for comparison with the older figures, and also to assemble in one table the most recent, and we believe the most accurate, figures for these two amino acids in the proteins that are listed.

EXPERIMENTAL.

Experience gained from numerous determinations of aspartic and glutamic acids in proteins leads us to regard as the most satisfactory the general analytical procedure described in the case of edestin. The description of the analysis of this protein is, therefore, given somewhat in detail.

The hydrolyses were conducted, except as otherwise stated, by first heating 50 gm. of the air-dried proteins with 200 cc. of 20 per cent hydrochloric acid on a steam bath until nearly all of the protein had dissolved. The hydrolyses were then continued by boiling the solutions in an oil bath for 30 to 36 hours.

Edestin.

Ether-extracted hemp seed meal was extracted with 5 per cent sodium chloride solution at 65°, and the mixture filtered while warm. The crystalline edestin that separated on cooling was twice recrystallized. It contained 4.70 per cent moisture, 0.12 per cent ash, and 18.84 per cent nitrogen.¹

Precipitation of Bases.—After hydrolysis, the suspended humin was filtered off on asbestos that had been previously thoroughly

¹ The percentages of nitrogen given in this paper for the proteins are calculated on an ash- and moisture-free basis.

digested with concentrated hydrochloric acid, and the dark solution was concentrated to a thick sirup under reduced pressure at a temperature not over 50° in order to remove as much as possible of the hydrochloric acid. The diamino acids were removed by precipitation with phosphotungstic acid. The precipitation and washing of the phosphotungstate precipitate were carried out in the manner usually followed in the Van Slyke method of analysis.

The filtrate from the phosphotungstate precipitate was freed from phosphotungstic acid by shaking with a mixture of equal volumes of amyl alcohol and ether, and the nearly colorless, aqueous solution of monoamino acids was concentrated to a thick sirup.

Precipitation of Barium Salts.—The sirupy residue was dissolved in 300 cc. of water, and moist, recrystallized barium hydroxide was added to the cooled solution until it was slightly alkaline to litmus. A small amount of brown, humin-like material separated. This was filtered off and washed several times with hot water. Barium hydroxide was then added to the solution until a small amount remained undissolved after shaking for a few minutes. The solution of barium salts (600 cc.) was poured with stirring into 5 times its volume of 95 per cent alcohol, and the mixture allowed to stand for 2 days.

The precipitated barium salts were washed with 95 per cent alcohol, then redissolved in 350 cc. of water and reprecipitated with 400 cc. of 95 per cent alcohol (1.14 volumes). After standing overnight in an ice box, the precipitate was filtered off by suction. Further addition of alcohol to the filtrate produced only a slight turbidity and on long standing (9 weeks) a light precipitate gradually settled. This product yielded, after quantitative removal of barium and chlorine, 1.19 gm. of tyrosine and 0.22 gm. of glutamic acid.

The alcoholic filtrate from the last small precipitate of barium salts was examined in order to ascertain how completely the dicarboxylic acids had separated by the second precipitation of barium salts, and also to learn what other amino acids had been precipitated by the first addition of the 5 volumes of alcohol. After quantitative removal of barium, and chlorine, the solution yielded 2.48 gm. of a solid, semicrystalline residue. Its aqueous solution was very slightly alkaline to litmus. It tasted sweet

and gave a strong Millon's test for tyrosine. Examination of this material by the carbamate method of Kingston and Shryver (4) showed that the greater part of the material was glycine. The second precipitation of the barium salts with a little more than an equal volume of 95 per cent alcohol, therefore, precipitated nearly all of the dicarboxylic acids, while most of the tyrosine and glycine, and a small quantity of bases that had been precipitated with the dicarboxylic acids by the first precipitation of barium salts, remained in solution.

Separation of Glutamic Acid.

The main precipitate of barium salts was decomposed with a slight excess of sulfuric acid, and the filtrate and washings from the barium sulfate were concentrated to about 800 cc. After quantitative removal of sulfuric acid the solution was evaporated to a small volume and saturated with hydrochloric acid gas. There was obtained in the usual way 7.91 gm. of pure glutamic acid hydrochloride. This together with the free acid obtained from the alcoholic filtrate from barium salts, and from the filtrate from the copper aspartate amounted to 9.12 gm. of glutamic acid, or 19.2 per cent of the edestin.

Separation of Aspartic Acid.

The filtrate and washings from the glutamic acid hydrochloride were concentrated to a sirup, in order to remove as much as possible of free hydrochloric acid. The remainder of the chlorine was removed by shaking the solution with an excess of silver sulfate. The solution yielded on evaporation a dry residue, none of which was soluble in glacial acetic acid.

The sirupy material usually associated with aspartic and glutamic acids at this stage of the analysis consists almost entirely of hydroxyglutamic acid and pyrrolidonecarboxylic acid, both of which are soluble in glacial acetic acid. The fact that this fraction of amino acids contained no acetic acid-soluble material demonstrates the absence of hydroxyglutamic acid in edestin. This is in accord with the results reported by Osborne, Leavenworth, and Nolan (12).

The dry residue of amino acids was dissolved in water, and the solution boiled with copper carbonate. The copper aspartate which separated in the characteristic crystalline form was washed several times with cold water. The air-dried copper salt weighed 9.91 gm. A further small quantity (0.14 gm.) was obtained from the filtrate. It contained 23.17 per cent of copper (calculated with $4\frac{1}{2}$ molecules of water, 23.07 per cent), and on heating at 125° and 25 mm. pressure lost water corresponding to $4\frac{1}{2}$ molecules. The free aspartic acid obtained by decomposing the copper salt with hydrogen sulfide behaved in the characteristic way on heating. It turned red but did not melt up to 300° . It contained 36.10 per cent carbon and 5.75 per cent hydrogen (calculated, C 36.07, H 5.30). The total amount of copper aspartate obtained corresponded to 4.85 gm. of aspartic acid, or 10.2 per cent of the edestin.

The filtrate from the copper aspartate after the removal of copper yielded by fractional crystallization 2.56 gm. of glutamic acid. The final filtrate which contained less than a gm. of solid material gave a strong Millon test for tyrosine.

Ovalbumin.

The ovalbumin was prepared from the whites of fresh hen's eggs according to the method described by Osborne and Campbell (13). It was twice recrystallized and consisted entirely of aggregates of small needles. The ovalbumin crystals were dissolved in a small quantity of water, and the protein was coagulated by pouring the solution into a large volume of boiling water. After being boiled for 15 minutes the coagulum was washed free from sulfates and dried with alcohol and ether in the usual way. The air-dried product contained 6.28 per cent of moisture, 0.11 per cent of ash, and 15.16 per cent of nitrogen.

The protein was hydrolyzed, and the diamino acids were removed as described in the case of edestin. A quantity (4.26 gm.) of glutamic acid was removed as the hydrochloride before precipitation of the barium salts. The barium salts of the dicarboxylic amino acid fraction were twice precipitated, first by addition of 5 volumes of alcohol, and then with 2 volumes. This fraction yielded no material soluble in glacial acetic acid, thus showing the absence of hydroxyglutamic acid in ovalbumin.

The alcoholic filtrate from the first precipitation of barium salts yielded 0.25 gm. of tyrosine and 0.63 gm. of glutamic acid. The filtrate from the second precipitation contained only a negligible amount of material. From the precipitated barium salts were obtained 2.91 gm. of aspartic acid, and 1.32 gm. of glutamic acid.

Altogether there were isolated 6.21 gm. of glutamic acid and 2.91 gm. of aspartic acid, equivalent, respectively, to 13.3 and 6.2 per cent of the ovalbumin.

Ox Muscle.

The material used for this analysis was obtained from a piece of lean, round steak. It was ground in a meat chopper and extracted successively, twice with distilled water, once with 50 per cent alcohol, and then with 95 per cent alcohol. After standing for 2 days in absolute alcohol, it was extracted with ether. The air-dried, nearly white material thus prepared contained 10.24 per cent of moisture, 0.33 per cent of ash, and 16.20 per cent of nitrogen.

The material was hydrolyzed, the diamino acids were removed, and the solution saturated with hydrochloric acid gas. There were obtained 3.49 gm. of glutamic acid hydrochloride. The barium salts of the remaining dicarboxylic amino acids yielded 8 gm. of amino acids insoluble in acetic acid, from which were obtained 2.62 gm. of aspartic acid, and 3.22 gm. of glutamic acid.

The total quantities of aspartic and glutamic acids isolated amounted, respectively, to 5.9 and 13.4 per cent.

Fish Muscle.

The skin, bones, and larger pieces of connective tissue were carefully removed from fresh halibut (*Hippoglossus hippoglossus*). The muscle was ground and extracted with water, alcohol, and ether as described in the case of ox muscle. The product contained 10.33 per cent of moisture, 0.77 per cent of ash, and 16.51 per cent of nitrogen.

The hydrolysis, precipitation of the bases, and removal of part of the glutamic acid (3.29 gm.) as the hydrochloride before precipitation of the calcium salts, were carried out as in the case of ox muscle. The dicarboxylic amino acids were then precipi-

tated as the calcium salts according to the method described by Foreman (2). The calcium was removed quantitatively with oxalic acid, the solution of amino acids freed from chlorine with silver sulfate, and concentrated to a small volume under reduced pressure. The residue was finally dried *in vacuo* over calcium chloride and triturated with glacial acetic acid until all soluble material had been removed. The insoluble portion (10 gm.) yielded in the usual way 3.54 gm. of aspartic acid and 1.03 gm. of glutamic acid.

Notwithstanding the precautions taken, as already referred to, to avoid conversion of glutamic acid to pyrrolidonecarboxylic acid, an appreciable quantity of the sirupy pyrrolidone compound was formed. Hydrolysis of this product by boiling with 25 per cent hydrochloric acid for 30 hours made possible, however, the recovery of 1.55 gm. of glutamic acid. There was also isolated 0.21 gm. of glutamic acid from the alcoholic filtrate from the calcium salts. All told, 6.08 gm. of glutamic acid and 3.54 gm. of aspartic acid were obtained, equivalent, respectively, to 13.7 and 8.0 per cent.

Arachin.

The arachin was first precipitated from a 10 per cent sodium chloride extract of finely ground, pressed Virginia peanuts by addition of ammonium sulfate to 30 per cent of saturation. The precipitate was dissolved by addition of water and reprecipitated by dialysis. After being washed and dried in the usual way, the protein contained 5.22 per cent moisture, 0.36 per cent ash, and 18.3 per cent nitrogen.

After removal of the diamino acids from the arachin hydrolysate, 6.83 gm. of glutamic acid were removed as the hydrochloride. The remainder of the dicarboxylic amino acids was precipitated as their barium salts. The free amino acids regenerated from the barium salts yielded 6.79 gm. of material insoluble in acetic acid, from which were isolated 2.64 gm. of aspartic acid.

The sirupy product dissolved by the acetic acid was boiled for 24 hours with 20 per cent hydrochloric acid. By precipitation of the barium salts 2.4 gm. of glutamic acid were recovered from this fraction in the usual way.

The total quantities of aspartic and glutamic acids isolated amounted, respectively, to 5.6 and 19.5 per cent of the arachin.

Glutenin.

Gluten, prepared by washing out the starch from a well known brand of wheat flour, was dried and the gliadin largely removed by extraction with 0.07 N acetic acid according to the method of Blish and Sandstedt (14). The residue was exhaustively extracted with 70 per cent alcohol, and then extracted with 0.2 per cent sodium hydroxide. The glutenin was precipitated by acidifying the alkaline extract with hydrochloric acid. It was purified by being redissolved in alkali and reprecipitated with acid. The air-dried protein contained 4.3 per cent moisture, 0.36 per cent ash, and 16.82 per cent nitrogen.

A quantity (33.3 gm.) of the glutenin equivalent to 31.8 gm. of ash- and moisture-free protein was hydrolyzed, and the barium salts of the dicarboxylic amino acid fraction were precipitated. Three precipitations of the barium salts were made, with use for the first precipitation of 5 volumes of alcohol, and 2 volumes for the other two. The barium salts yielded 10.17 gm. of amino acids insoluble in acetic acid, from which were isolated 8.16 gm. of glutamic acid, and 0.65 gm. of aspartic acid, equivalent, respectively, to 25.7 and 2.0 per cent of the glutenin hydrolyzed.

Gliadin.

Gliadin was prepared by extracting wheat gluten with 70 per cent alcohol, and pouring the clear alcoholic extract into several volumes of water containing a small quantity of lithium chloride. The precipitated protein was purified by two successive precipitations with absolute alcohol. After being dried in the usual way, it contained 6.79 per cent moisture, 0.19 per cent ash, and 17.87 per cent nitrogen.

Three separate hydrolyses were made of different lots (Lots A, B, and C) of gliadin containing quantities equivalent to 46.5, 46.5, and 272.5 gm., respectively, of ash- and moisture-free protein. After hydrolysis of Lot A the dicarboxylic acid fraction was precipitated as the calcium salts, and the solution of the regenerated amino acids saturated with hydrochloric acid gas and as much glutamic acid hydrochloride removed as possible. The subsequent procedure was carried out in the usual order, removal of the fraction soluble in acetic acid, separation of aspartic acid as

the copper salt, and of the remaining glutamic acid by fractional crystallization. The same procedure was followed in the analysis of Lot B, with the exception that barium hydroxide was used instead of calcium hydroxide. In the case of Lot C, as much glutamic acid as possible was removed as the hydrochloride directly after hydrolysis. The remainder of the dicarboxylic amino acids was precipitated as the barium salts. The quantity of diamino acids in gliadin is so small that the precipitation with phosphotungstic acid was considered unnecessary.

The analyses gave the following percentage results: *Glutamic acid*:² Lot A, 41; Lot B, 41.15; Lot C, 40.69. *Aspartic acid*: Lot A, 0.77; Lot B, 0.57; Lot C, 0.64.

Each lot yielded considerable material soluble in acetic acid, which, for the greater part, doubtless consisted of hydroxyglutamic acid. The presence of this amino acid in gliadin has been previously demonstrated by Dakin (15).

Glycinin.

The glycinin was prepared from ground yellow soy beans as described by Osborne and Campbell (16). The preparation had the following percentage composition: ash, 0.83; moisture, 6.68; nitrogen, 17.04.

From the hydrolysate of a quantity of the glycinin equivalent to 98.6 gm. of the ash- and moisture-free protein, 14.58 gm. of glutamic acid were removed directly as the hydrochloride. The diamino acids were then precipitated with phosphotungstic acid, and the dicarboxylic amino acids separated as their calcium salts. The calcium salts yielded 17.0 gm. of material insoluble in acetic acid, from which were isolated 9.26 gm. of aspartic acid, and 3.61 gm. of glutamic acid. The percentage yields of aspartic and glutamic acids were 9.4 and 18.5, respectively.

SUMMARY.

The percentages of aspartic and glutamic acids in proteins as recorded in the literature are in nearly all cases too low, with the

² Since the work described in this paper was completed, 43.0 per cent of glutamic acid has been isolated from gliadin by Russell Wilson of this laboratory in a special study of the dicarboxylic amino acids of this protein. This is in close agreement with the percentage previously found by Osborne and Guest (17).

exception of those for three or four proteins which have been recently analyzed by newly developed methods. Particularly is this true for aspartic acid. New determinations of these amino acids have been made in a number of typical proteins, and invariably higher results for aspartic acid were obtained than those previously obtained by the ester method. In several cases from 4 to 9 times as much was found. In the case of glutamic acid such wide differences were not found, especially in those cases where the old figures had been obtained chiefly by the separation of glutamic acid hydrochloride directly from the hydrolysates, and where the losses attendant upon the ester method of analysis were not involved.

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THE PHYSIOLOGICAL RESPONSE OF RABBITS TO INSULIN.

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Several papers dealing with the physiological response of rabbits to insulin have appeared. It would seem unnecessary to add still another were it not for the fact that the data presented in the literature are not strictly comparable. This situation is due to the large variability in response to insulin shown by rabbits. We have in our possession a considerable number of animals whose response to the intravenous injection of insulin is known within certain well defined limits. These "standard" rabbits have afforded a good opportunity of studying certain of the factors which influence the sensitivity or, *vice versa*, the resistance of rabbits to insulin. We have compared the response to intravenous, subcutaneous, and intraperitoneal injections of insulin, and have also investigated some of the interrelations of epinephrine and insulin. Further observations on the production of immunity to insulin were also made.

Intravenous, Subcutaneous, and Intraperitoneal Injections of Insulin.

The purpose of this experimental work on the intravenous, subcutaneous, and intraperitoneal injections of insulin into standard rabbits is to demonstrate clearly that: (1) more insulin is required when given intravenously than in the case of either of the other methods; (2) the time necessary to produce convulsions is less after intravenous injection than after the other two forms; (3) and the period of hypoglycemia is longest after subcutaneous and shortest after intravenous injections.

In this experiment seventeen of our standard rabbits which had been used for insulin standardization were selected. Some of

these had been used for this purpose for over 3 years. Their seasonal variation is not very large. Nevertheless, their intravenous factor of resistance is carefully watched. It is of interest to note that whenever the intravenous factor varied, the subcutaneous and the intraperitoneal factors varied also. These standard animals, as mentioned elsewhere (1), are kept on a strict regimen and given convulsive doses every 7th day. The insulin

TABLE I.

Intravenous, Subcutaneous, and Intraperitoneal Convulsive Doses of Insulin.

Rabbit No.	Intravenous injection.		Subcutaneous injection.		Intraperitoneal injection.	
	Convulsive dose.	Time.	Convulsive dose.	Time.	Convulsive dose.	Time.
	<i>units per kg.</i>	<i>min.</i>	<i>units per kg.</i>	<i>min.</i>	<i>units per kg.</i>	<i>min.</i>
26	1.4	95	0.9	150*	0.9	150*
114	1.3	80	0.5	176	0.5	140
112	1.2	75	0.6	195	0.5	144
32	1.2	76	0.6	120	0.7	124
2	1.0	85	0.4	155	0.5	128
1	1.0	70	0.5	165	0.5	132
29	1.0	74	0.7	265	0.7	175
23	0.8	55	0.4	169	0.4	125
33	0.8	76	0.5	128	0.5	255
120	0.8	99	0.6	145	0.6	95
99	0.7	75	0.6	118	0.6	165
121	0.6	120*	0.45	180*	0.45	180*
77	0.6	95	0.4	124	0.4	90
12	0.55	55	0.25	132	0.25	120
78	0.5	48	0.3	80	0.3	84
64	0.5	65	0.5	157	0.5	135
39	0.45	40	0.15	90	0.2	95

* Approximately.

used for these experiments was Lilly's U-100, chosen of one lot number, and diluted 1:100. Blood was removed from the marginal ear vein, and the sugar determinations were made according to the method of Folin and Wu. Our comparative results for the different forms of injection are found in Table I.

The rabbits are arranged in order, beginning with the highest intravenous factor of resistance. In Table I, the figures were not regarded as the lowest convulsive doses until the next lowest

doses were tried the following week, and negative results were obtained, after which they were checked again. The time for convulsion in every case is reported for the last check made, and in most cases it proved to be as previously observed. It is plain from the data that the intravenous factor exceeds both the subcutaneous and the intraperitoneal. Only Rabbit 64 gave an identical figure of 0.5 unit per kilo in the three cases, but the time required for convulsions was different in each case. Rabbit 39 showed the least resistance to insulin following the different forms of its administration. With larger doses of insulin, intravenously injected, it had convulsions in 35 minutes. A comparison between the intravenous and the subcutaneous factors of this animal shows that there is a difference of 300 per cent, while between the subcutaneous and the intraperitoneal factors the difference is negligible. In most instances, the subcutaneous and the intraperitoneal factors were practically identical, while the variation between these two factors taken as one unit and the intravenous factor was large. No definite ratio could be established which would enable one to draw a general conclusion. The individual variation of factors, as can be clearly seen, varies so much that in order to study this comparison an individual study should be made.

It is, however, worthy of observation that although these animals readily respond to insulin, yet they have a tendency, once standardized, to respond to subconvulsive doses of insulin in a uniform manner as regards the duration and the degree of hypoglycemia. A few of our standard rabbits were chosen at random to demonstrate this observation. The conditions were slightly altered. After a 24 hour fast, the animals were given very small amounts of insulin (U-100 was used and diluted 1:200). An initial blood sample was taken from the marginal ear vein, after which insulin was injected intravenously. 75 minutes later another sample of blood was removed. The rabbit was then kept unfed for another 24 hours. The same quantity of insulin was injected intravenously and the blood sample, both before and 75 minutes later, was removed exactly as on the previous day. Our results for this experiment are reported in Table II. This experiment shows very clearly that standard animals become very sensitive to insulin. 0.1 unit per kilo, injected intravenously, lowers the blood sugar appreciably.

In the third experiment of this series, three of our standard rabbits were chosen to demonstrate the blood sugar curve that may be produced after the administration of small amounts of insulin. Subminimal convulsive doses of insulin were injected intravenously, subcutaneously, and intraperitoneally. Blood samples were removed every 15 minutes during the 1st hour after each injection and then every half hour for a period of about 5

TABLE II.
Hypoglycemia in Standard Rabbits Produced by Intravenous Injection of Subconvulsive Doses of Insulin.

Rabbit No.	Date.	Weight.	Units given per kg.	Blood sugar per 100 cc.		Intravenous convulsive dose.	Time for convulsion.
				Initial.	75 min.		
	1927	kg.		mg.	mg.	units per kg.	min.
32	Nov. 7	3.35	0.15	100	83	1.2	76
	" 8	3.35	0.15	103	85		
	Dec. 5	3.35	0.3	105	85		
33	" 6	3.35	0.3	105	77		
	Nov. 7	3.25	0.15	118	65	0.8	76
	" 8	3.25	0.15	114	69		
	Dec. 5	3.00	0.3	118	35		
	" 6	3.00	0.3	118	42		
15	Nov. 7	3.25	0.15	108	65	1.2	74
	" 8	3.25	0.15	108	71		
	Dec. 5	3.25	0.3	111	65		
	" 6	3.25	0.3	108	70		
2	Nov. 7	3.50	0.15	100	68	1.0	85
	" 8	3.50	0.15	100	74		
	Dec. 5	3.40	0.3	105	50		
6	" 6	3.40	0.3	111	55		
	Nov. 9	2.60	0.1	118	59	0.4	53
	" 10	2.60	0.1	121	60		

hours. In the case of both the subcutaneous and intraperitoneal injections, there was no lowering of blood sugar after the first 15 minutes, while after the intravenous injection the blood sugar fell in the three cases, as shown in Fig. 1. The duration of hypoglycemia was the longest after the subcutaneous injections and the shortest after the intravenous injections. Insulin intraperitoneally injected was as effective as when given subcutaneously,

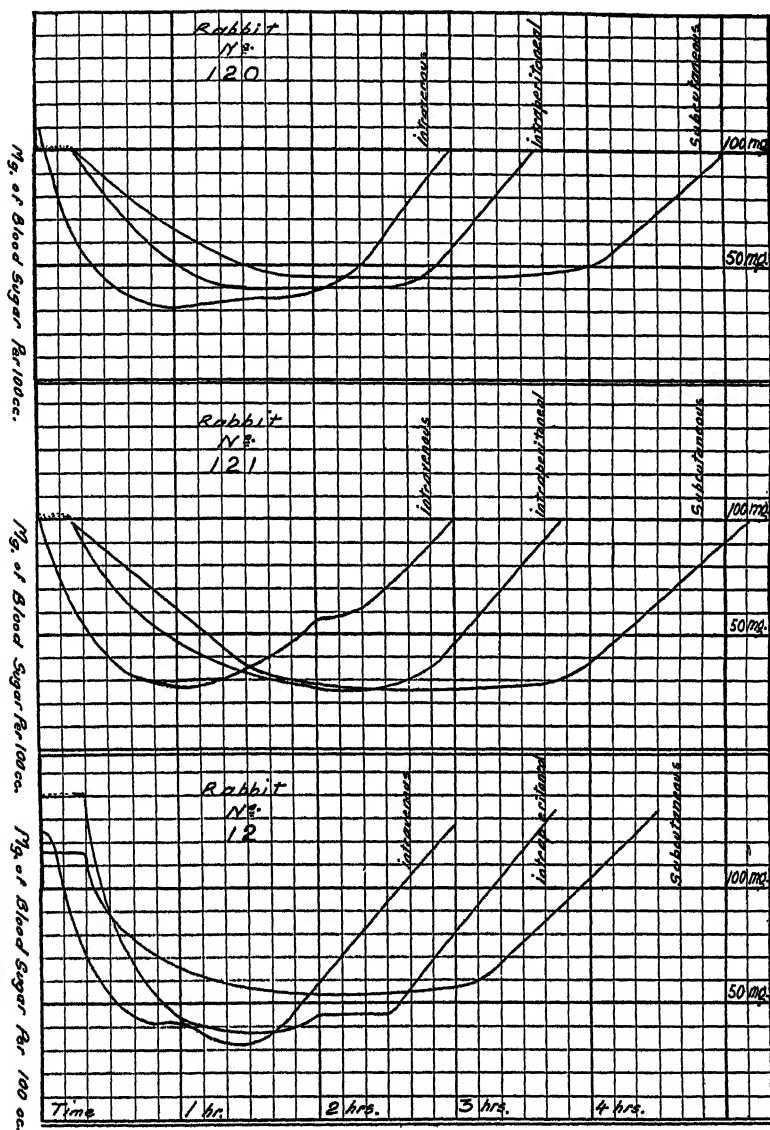


FIG. 1. Blood sugar curves of standard rabbits following the injection of subconvulsive doses of insulin.

but as to the duration of hypoglycemia the results showed an intermediary position.

As an illustration of the constancy of response to insulin in the case of standard rabbits, the results with Rabbit 12 are cited. 53 minutes after the intravenous injection of 0.5 unit per kilo the rabbit had a slight convulsion and the lowest blood sugar (32 mg. per 100 cc.) was recorded at 92 minutes. 2 weeks later the same dose was repeated. The slight convulsion occurred at 51 minutes and the lowest sugar (30 mg. per 100 cc.) was recorded at 93 minutes.

Other items, such as the intravenous, subcutaneous, and intra-peritoneal factors of resistance, time for convulsion, *etc.*, are reported in Table I.

Epinephrine and Insulin.

Our experiments with epinephrine and insulin were planned to secure additional evidence in support of the hypothesis that the liver glycogen plays an important rôle in the resistance of animals towards insulin, and that this glycogen is mobilized after the injection of insulin. In the foregoing experiments, it was shown that a quantity of insulin as low as 0.1 of 1 unit lowers the blood sugar appreciably, so that in order to consume the sugar reserve in the blood a very small amount of insulin is required. But when one encounters the fact that two or three times that quantity, and often more, is required to produce convulsions and yet maintain the same low level of blood sugar, one is puzzled and tries to find a cause for this increased requirement of insulin.

In the foregoing experiments, no attempt was made to explain the reason or reasons for the difference which exists between the response to intravenous and subcutaneous injections. A plausible hypothesis which explains these phenomena follows. After the intravenous injection of insulin the liver is indirectly stimulated by the comparatively large amount of insulin in the blood stream, and the action of insulin so injected is consequently less powerful than when otherwise administered. More glycogen is mobilized to counteract its effect, and in order to produce convulsions additional insulin must be injected. According to a paper from this laboratory (2), Barbour, Chaikoff, MacLeod, and Orr (3), and Dudley and Marrian (4), liver glycogen in fasting rabbits exists in an average of 0.19 gm. per 100 gm. of liver weight.

If A represents the amount of insulin required to lower the blood sugar of a rabbit from 100 to 45 mg. per 100 cc. without causing convulsions, and A' represents the entire amount of insulin required to produce convulsions, the difference between A' and A is usually several times greater than A itself. Let us call this difference B . B seems to us to represent not only that difference in amount of insulin which is required to bring about convulsions, but to indicate the resistance of animals toward insulin. If all preparations of insulin were alike, and the constituents of the bodies of different animals were similar, A would be constant. Hence, the quantity of insulin required to lower the blood sugar from 100 to 45 mg. would be the same in most cases. But it seems that it is almost impossible to control all the varying factors no matter how careful our standardizations may be or how accurate we may be in our determinations. Insulin, though in small quantities, seems to excite the liver when injected intravenously, and force it to mobilize some of its glycogen. Were we to neglect this small quantity of mobilized glycogen which is caused by A , $A' - A$, which we have said is equal to B , would probably represent also the amount of insulin required to care for the glycogen liberated from the liver. Glycogen does not exist in equal amounts per 100 gm. of liver weight in all animals, so that the amount that might be mobilized when insulin is injected is never the same. It is, therefore, evident that B is not the same in all rabbits. It follows, also, assuming that our deduction is correct, that resistance in rabbits is different—which is a fact known to every insulin worker.

It remains to be seen whether B could or could not represent also the glycogen mobilized to resist the effect of insulin, or, reversing it, whether or not most of the resistance in rabbits depends upon the amount of liver glycogen mobilized following the intravenous injection of insulin. Upon reviewing our experiments dealing with the intravenous, subcutaneous, and intraperitoneal injection of insulin into rabbits, one observes, first, that the subcutaneous and intraperitoneal factors are practically identical; second, that although they are practically identical, yet the time for convulsion resulting from intraperitoneal injections is different from the subcutaneous. These findings would justify our assumption that the process by which insulin is absorbed after

subcutaneous injections is slower than after the intraperitoneal. However, the fact that the quantity is practically identical shows that after such injections the mobilization of the liver glycogen is not inordinately increased as occurs after the intravenous administration.

This hypothesis that there is more liver glycogen mobilized after intravenous injection of insulin into animals than after other forms of parenteral administration, needs further support. Injection of epinephrine into animals is known to cause hyperglycemia, aside from its various other physiological effects. This hyperglycemia, which persists for several hours, is caused by the mobilization of liver glycogen. Part of the sugar thus formed is recovered in the urine, and another part is perhaps oxidized, Cori and Cori (5). What becomes of the rest of the sugar we do not attempt to explain. What concerns us in this matter is to decrease the amount of glycogen reserve in the liver. By so doing, if our hypothesis is correct, the resistance in animals towards insulin should diminish and the administration of epinephrine should permit the production of a hypoglycemia with smaller amounts of insulin than would otherwise be required.

Nine of our standard rabbits were used for this experiment. After a 24 hour fast, blood samples were removed for sugar tests. Insulin was administered intravenously in very small quantities, beginning with 0.1 to 0.2 unit per kilo. 60 to 75 minutes later samples of blood were again removed. The injection of such small quantities of insulin was meant to produce a noticeable hypoglycemia which could be rendered more so either by increasing the quantity of insulin or by other means. These animals were then kept in a fasting condition another 24 hours. They were further subjected to the same treatment of the day before for checking purposes. A 48 hour fast did not render these animals more sensitive than a 24 hour fast. Our results are reported in Table III. 1 week later the rabbits were fasted for 24 hours and then epinephrine was administered subcutaneously in doses of 2 mg. This quantity was given in two doses with an interval of 1 hour between. Another period of 24 hours of fasting followed and then insulin was administered in the same quantities used the week before. Samples of blood were removed also in a similar manner and at identical intervals. See Table III for results

TABLE III.

Effect of Previous Injections of Epinephrine on Hypoglycemia Produced by Small Amounts of Insulin.

Date.	Rabbit No.	Weight.	Insulin.	Blood sugar per 100 cc.		Date.	Insulin.	Blood sugar per 100 cc.		Epinephrine.
				Initial.	75 min.			Initial.	75 min.	
1927		kg.	units per kg.	mg.	mg.	1927	units per kg.	mg.	mg.	mg.
Nov. 7	32	3.35	0.15	100	83	Nov. 14				2
" 8	32	3.35	0.15	103	85	" 15	0.15	85	45	
" 21	32	3.35	0.25	100	95	" 28				2
" 22	32	3.35	0.25	100	91	" 29	0.25	105	45	
" 7	33	3.25	0.15	118	65	" 14				2
" 8	33	3.25	0.15	114	69	" 15	0.15	105	39	
" 21	33	3.25	0.25	125	61	" 28				2
" 22	33	3.25	0.25	100	55	" 29	0.25	103	22*	
" 7	15	3.25	0.15	108	65	" 14				2
" 8	15	3.25	0.15	108	71	" 15	0.15	105	50	
" 21	15	3.25	0.25	108	66	" 28				2
" 22	15	3.25	0.25	118	63	" 29	0.25	103	45	
" 7	2	3.50	0.15	100	68	" 14				2
" 8	2	3.50	0.15	100	74	" 15	0.15	91	38	
" 21	2	3.50	0.25	110	71	" 28				2
" 22	2	3.50	0.25	100	60	" 29	0.25	105	25*	
" 9	6	2.60	0.10	118	59	" 16				1
" 10	6	2.60	0.10	121	60	" 17	0.10	110	31	
						" 30				1
						Dec. 1	0.20	100	20†	
" 9	7	3.25	0.30	100	62	Nov. 16				2
" 10	7	3.25	0.30	111	62	" 17	0.30	100	45	
" 18	19	4.00	0.15	108	73	Dec. 2				3
" 19	19	4.00	0.15	105	74	" 3	0.15	100	65	
" 18	114	3.65	0.20	111	69	" 2				3
" 19	114	3.65	0.20	108	70	" 3	0.20	114	41	
" 18	112	4.50	0.20	108	64	" 2				3
" 19	112	4.50	0.20	125	67	" 3	0.20	121	55	

* Almost convulsed.

† Severe convulsions.

It is obvious that the injection of epinephrine 24 hours before the administration of insulin had appreciably decreased the resistance of the standard rabbits to insulin. It seems probable, therefore, that the liberation of liver glycogen the day before had caused this

noticeable effect. One has to consider also the possibility that some of the insulin intravenously injected may be excreted in the urine.

It is also interesting to note that repeated injections of epinephrine into standard rabbits seem to produce comparable hyperglycemic results. Such results are to be expected when the liver glycogen remains constant. Epinephrine was given to Rabbits 112 and 114; samples of blood were taken for sugar determinations at 1.5, 4, and 5 hours. An equal amount of epinephrine was given them 1 week later and a check for blood sugar was made. See Table IV for results.

TABLE IV.

Effect of Repeated Injections of Epinephrine on Blood Sugar of Standard Rabbits.

Date.	Rabbit No.	Weight.	Epinephrine.	Blood sugar per 100 cc.			
				Initial.	1.5 hrs.	4 hrs.	5 hrs.
1927		kg.	mg.	mg.	mg.	mg.	mg.
Dec. 2	114	3.65	3	114	278	348	374
" 9	114	3.65	3	105	295	340	365
" 2	112	4.50	3	95	334	374	420
" 9	112	4.50	3	100	340	380	400

The Effect of Splanchnectomy upon Resistance to Insulin.

In the preceding experiments with epinephrine and insulin it was shown that by the subcutaneous injection of small quantities of epinephrine into reliable standard rabbits, 24 hours before insulin was administered, their resistance toward the latter is diminished. Other workers (5) have demonstrated that by removing the suprarenals in rats the sensitivity of these animals toward insulin is greatly increased. Because rabbits are not entirely suitable subjects for suprarenalectomy, different methods similar to those adopted by Britton, Geiling, and Calvery (6) were followed for the further study of the physiological action of insulin. If epinephrine from natural sources stimulates the mobilization of liver glycogen and impedes the effect of insulin, its absence should therefore increase the effect of the latter.

Four male and five female rabbits, whose splanchnic nerves running to the suprarenals had been severed, were used. As

reported previously (1) young "uneducated" rabbits are very resistant to insulin and convulsions do not occur unless several units per kilo of body weight are administered. Rabbits 404 and 406 of this group are the only ones that had previously been given insulin. That was 6 months before splanchnectomy was done. Their diet during the 6 months of freedom was the high carbohydrate diet consisting of barley and alfalfa hay every day. Consequently their resistance to insulin was naturally increased, as was demonstrated (2) and as will be shown later in this paper. From the many observations which we have made,

TABLE V.
Intravenous and Subcutaneous Convulsive Doses of Insulin in Splanchnectomized Rabbits.

Rabbit No.	Weight.	Intravenous injections.			Subcutaneous injections.			Sex.
		Date.	Units per kg.	Time for convulsion.	Date.	Units per kg.	Time for convulsion.	
	kg.	1928		min.	1928		min.	
0	3.00	Jan. 23	0.5	60	May 4	0.4	120	Male.
404	3.20	Feb. 25	0.5	65	" 4	0.3	120	Female.
406	2.80	Apr. 27	0.4	55	" 18	0.25	95	"
40	2.50	Mar. 29	0.5	92	" 2	0.3	143	Male.
41	2.50	" 29	0.6		" 2	0.5	133	"
42	2.50	May 23	0.4	55	" 2	0.25	91	"
43	2.30	" 21	0.6	73	" 21	0.45	92	Female.
44	2.30	Apr. 16	0.4	58	" 1	0.4	82	"
45	2.50	" 16	0.4	61	" 1	0.4	62	"

it may be safely said that no young uneducated rabbit had convulsions with a dosage of less than 3 units per kilo. Larger quantities of insulin were usually required for this purpose. It may therefore be said to be true of this group of animals. Several weeks after the operations, when the animals had completely recovered, they were put on the low carbohydrate diet. A 24 hour fast preceded the administration of insulin. A dose of 0.5 unit was tried and severe convulsions followed in most cases. Upon further trials their lowest convulsive doses were noted. See Table V for results. The remarkable manner in which these animals respond to insulin, the facility of finding the lowest

convulsive doses, and their reliability (not depending upon the essential 7th day convulsive dose), lead us to suggest this as a new and reliable method for the physiological assay of insulin.

This decrease of resistance to insulin in rabbits whose splanchnic nerves are severed, acts as a further support to the theory that the mobilization of the liver glycogen increases resistance. Also, the inability of the liver to mobilize its glycogen when insulin is injected decreases the resistance of the animals. Epinephrine seems to be, therefore, an essential factor in the carbohydrate metabolism, and to control to a certain extent, one of the strongest factors of resistance in animals to insulin.

In this connection we might say that it cannot be definitely stated whether or not all of these animals had a complete splanchnectomy. Had there been a complete severance of the splanchnic nerves, we are inclined to believe that the convulsive intravenous doses of insulin, as well as the subcutaneous would be uniform. The ratio between the two would be a more or less definite one since a great obstacle to insulin in the body would be removed.

Immunity to Insulin in Rabbits.

Since reporting the cases, 2 years ago, of the two rabbits which developed immunity to insulin (1), we have received some inquiries about their blood sugar before and after the injection of insulin. Unfortunately complete data could not then be given as our problem dealt mostly with the assay of insulin and by chance we came across the two immune rabbits, Rabbits 92 and 93. As no one has added further information on the subject it was thought advisable to repeat this experiment administering larger doses of insulin than had previously been used.

Three male and two female rabbits were chosen for this problem. All of these animals were used in our experiments dealing with the effect of intraperitoneal injections of insulin upon the blood sugar of well fed rabbits (7). They were kept, as mentioned therein, on the high carbohydrate diet. The animals used in the experiments just referred to, required no period of fasting before the administration of insulin. In this instance however, a different method was employed. The three males and two females, Rabbits 1, 2, 4, 54, and 57, were found to be very suitable animals

for illustrating immunity to insulin. They were kept on the same diet and a 24 hour fast preceded every injection of insulin. The largest quantity of insulin injected into these animals up to January 26, 1928, was 8 units per kilo. On February 2, the present series of experiments was started. Lilly's insulin U-40 (undiluted) was first used in total amounts of 40 units or less. In larger quantities, Lilly's U-100 was employed, undiluted also. All injections were made intravenously. 10 units per kilo were first administered and this dose was gradually increased every week. Naturally, the increase at first was slow, as no convulsions were desired. The increasing dosage also depended upon the behavior of the animals the week before. Had convulsions occurred, there would have been little hope of attaining the remarkable immunity recorded in Tables VI and VII. Blood was removed before the administration of insulin and later when the animals showed signs of weakness. This weakness was evidently due to the large amounts of insulin injected. Experiments on Rabbit 1 had to be discontinued for a few weeks to allow it to recover from an infection in its back caused by bites inflicted by other rabbits. However, it was able to withstand later a total amount of 82 units. Rabbits 2 and 3 withstood each 90 and 100 units, respectively, without convulsions. The fact that they were able to resist such large quantities of insulin without convulsion and to recover without the assistance of glucose injections is a remarkable illustration of immunity to insulin. The behavior of these animals after these injections is noteworthy. They were never completely unconscious as occurs in other animals when in such a condition. When barley was offered to them under such circumstances they were able to distinguish it and to nibble their food. A few minutes later they regained their activities and seemed as lively as if insulin had never been administered. Manifestations of hypoglycemia were observed when these experiments were first started and later when the largest doses were given. These were observed 45 minutes after the administration of insulin and lasted for over 1 hour. The animals then completely recovered and showed an excellent appetite.

The maximum quantities that were tried with Rabbits 54 and 57 were 90 and 85 units respectively. Data on the various doses of insulin are reported in Table VII. With these two animals

a series of experiments were carried on to demonstrate the blood sugar curve caused by large and small doses of insulin. Into Rabbit 54 the following total amounts were injected: 50, 25, 12.5, and 4 units. To Rabbit 57 somewhat different quantities were administered, as follows: 50, 10, 5, and 2.5 units (total). Blood samples for sugar determinations were removed every 15 minutes the 1st hour and then every 30 minutes for about 5 hours. Our results are represented by the curves plotted in Fig. 2.

We make no attempt at the present time to offer any explanation of immunity in rabbits. These observations reported are merely to show that immunity to insulin can be developed to a high degree. Also that the insulin administered lowers the blood sugar of these animals. Whether the injections are small or large the duration of hypoglycemia is not in any definite ratio to the quantity of insulin given intravenously.

TABLE VI.
Insulin Hypoglycemia in Immune, Male Rabbits.

Date.	Rabbit 1.					Rabbit 2.				
	Weight.	Insulin units.		Blood sugar per 100 cc.		Weight.	Insulin units.		Blood sugar per 100 cc.	
		Per kg.	Total.	Initial.	Low.		Per kg.	Total.	Initial.	Low.
	kg.			mg.	mg.	kg.			mg.	mg.
Feb. 2	2.60	10	26			2.20	10	22		
" 9	2.60	12	30.5	105	37	2.20	12	25	100	30
" 16	2.60	13	34	91	36	2.20	13	28.5	95	40
" 23	2.60	18	45			2.20	15	31.5	105	44
Mar. 1	*					2.20	20	44	90	33
" 8	*					2.20	22.5	50	87	47
" 15	*					2.20	25	55	95	40
" 22	*					2.20	30	65	105	37
" 29	*					2.20	35	75	87	33
Apr. 5	2.55	25	65	98	40	2.20	40	90	100	30†
" 12	2.75	30	82	83	34	2.20	10	22		†
" 19	2.75	30	82	†						

* This rabbit was not given any insulin from March 1 to 29. It received an infection caused by bites.

† Convulsions.

‡ Weakness at 1 hour after the injection of 90 units. An hour later 5 cc. of 20 per cent glucose were administered subcutaneously and the animal recovered immediately.

TABLE VI—*Concluded.*

Date.	Rabbit 4.					
	Weight.	Units of insulin.		Blood sugar per 100 cc.		
		Per kg.	Total.	Initial.	Low.	
	kg.			mg.	mg.	
Feb. 2	2.35	10	23.5			
" 9	2.35	12	27.5	95	38	
" 16	2.35	13	31	100	40	
" 23	2.35	15	36	95	48	
Mar. 1	2.50	20	50	87	37	
" 8	2.50	22.5	56	85	55	
" 15	2.50	25	61	87	59	
" 22	2.50	30	72	87	50	
" 29	2.50	35	85	93	47	
Apr. 5	2.50	40	100	93	47	
" 12	2.50	40	100	89	35	
" 19	2.50	40	100			Convulsions in 71 min.
" 26	2.50		70			" " 105 "
May 3	2.50		40			" " 90 "
" 10	2.50		30			No convulsions.

TABLE VII.

Insulin Hypoglycemia in Immune, Female Rabbits.

Date.	Rabbit 54.						Rabbit 57.					
	Weight.	Units of insulin.		Blood sugar per 100 cc.			Weight.	Units of insulin.		Blood sugar per 100 cc.		
		Per kg.	Total.	Initial.	1 hr.	2 hrs.		Per kg.	Total.	Initial.	1 hr.	2 hrs.
	kg.			mg.	mg.	mg.	kg.			mg.	mg.	mg.
Feb. 24	4.00	7	28				3.50	7	24.5			
Mar. 1	4.00	10	40	100	39	30	3.65	10	36	108	38	30
“ 8	4.00	10	40	110	43	30	3.65	15	55	100	31	30
“ 15	4.00	12.5	51	100	40		3.65	15	55	100	33	
“ 22	4.10	15	62	95	41		3.70	17.5	65	93	43	
“ 29	4.10	15	62	111	40		3.75	17.5	65	105	37	
Apr. 5	3.95	20	77	105	63		3.70	20	75	105	50	
“ 12	3.95	22.5	90	118	33		3.75	22.5	85	105	33	

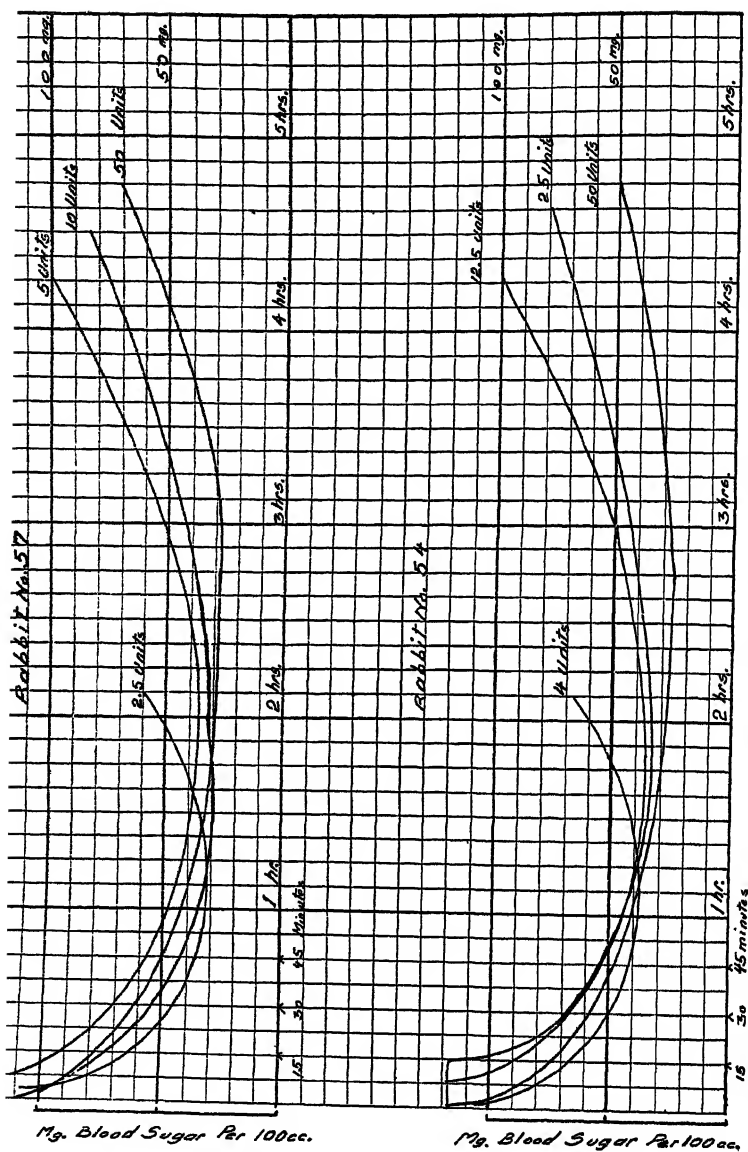


Fig. 2. Blood sugar curves of immune rabbits following the intravenous injection of small and large amounts of insulin

A few more experiments were carried out to find how long the insulin administered stayed in the blood stream. Though the results obtained are not sufficient to justify a definite conclusion, yet they lead us to believe that approximately 2 hours after the intravenous injection of large doses, insulin almost vanishes from the blood stream of these animals.

SUMMARY.

1. More insulin is required to produce convulsions when given intravenously than when administered either subcutaneously or intraperitoneally. The time necessary to produce convulsions is less after intravenous injections than after the other two forms. The intraperitoneal route seems to be on a par with the subcutaneous.

2. A hypothesis is presented and discussed in explanation of the differences that exist between the effects of the subcutaneous and the intravenous injections of insulin. Insulin intravenously injected is thought to stimulate to a greater degree the mobilization of liver glycogen. Also it appears likely that the mobilization of liver glycogen is an important factor in the resistance of animals to insulin.

3. Epinephrine injections 24 hours before insulin administration render the animals more sensitive to insulin. The fact that epinephrine liberates liver glycogen may explain this phenomenon.

4. Experiments with splanchnectomized rabbits show that animals whose splanchnic nerves are severed become very sensitive to insulin. It is suggested that such animals might advantageously be used for the assay of insulin.

5. Immunity was developed to a marked degree in rabbits fed a high carbohydrate diet. Blood sugar curves resulting from the injection of such large amounts of insulin are plotted. The method followed in developing immunity in rabbits is described. No explanation of this immunity is offered.

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VITAMIN A DEFICIENCY AND CALCIFICATION OF THE EPITHELIUM OF THE KIDNEY.

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PLATE 1.

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Recently¹ I gave an account of an investigation into the occurrence of urinary calculi in rats on a vitamin A-deficient diet, and this investigation led me to the conclusion reached by Osborne and Mendel;² namely, that there is a relation between a certain form of calculosis and vitamin A deficiency.

In the course of the investigation I frequently found in these rats, besides calculi in the bladder, also microscopic concretions agreeing in form with casts, but of crystalline nature. Of such casts a photographic plate was published. This result led me also to examine the kidneys of rats on a vitamin A-deficient diet. I repeatedly found tubules, convoluted and looped as well as straight ones, here and there containing a substance soluble in acid, which turned black in silver nitrate and blue with hematoxylin, so that it might be taken for a calcium salt. In the report there was also a photograph of a kidney section stained with silver nitrate in which there were many such concretions.

This calcium deposit so frequent with vitamin A deficiency that it may be considered a pathognostic sign of it, throws light on the origin of the calculi found by Osborne and Mendel in the bladder of the laboratory rat. It is very probable, not to say certain, that such calcium deposits become loosened and are washed by the urine into the bladder, where, as corpora aliena, they increase in size by means of further deposition of salts from the urine.

In my former communication I had suggested the possibility

¹ van Leersum, E. C., *J. Biol. Chem.*, 1928, lxxvi, 137.

² Osborne, T. B., and Mendel, L. B., *J. Am. Med. Assn.*, 1917, lxi, 32.

that we have here a morbid change of epithelial cells which gives rise to the formation of calcium casts. Deficiency of vitamin A frequently causes changes in epithelial tissue, as xerophthalmia and the disturbance of the vaginal secretion in estrus show. Therefore I examined these calcium deposits in the kidney tubules microscopically. A description of what I found follows.

When examining many preparations one is apt to come across calcium deposits which do not appear homogeneous; for instance, if the action of the silver nitrate solution has been of short duration and they have not been long exposed to the light. They then appear to consist of lumps which differ in depth of color. Some are irregular or square in form (Figs. 1 and 5), others round or oval (Fig. 2). They are of the same size as the epithelial cells of the tubules. Though hardly anything is to be seen of structure, some possess a sort of nucleus differing in coloring and the refraction of light from the rest of the deposit (Fig. 3). These lumps are arranged in a particular way. If by chance the section has been made through the longitudinal axis of such a cast, they can be seen to lie in two rows on either side of the wall of the tubule (Fig. 5). That they do not entirely fill the lumen becomes evident from the concretions cut perpendicularly on the axis, which assume the shape of a ring (Figs. 1 to 3). In other places dark round or oblong spots are seen without recognizable structure (Figs. 1 to 3) and sometimes lying in an entirely or partly closed circle (Fig. 1, 4). These spots are of the same size as nuclei. All this can be seen still better in preparations stained for only a short time with hematoxylin. In unstained sections the calcium casts can also be distinguished from apparently normal tubules, owing to a stronger refractive power and also owing to the fact that remains of tissue elements can still be distinguished in the calcified matter. This appears from the two photographs (Figs. 4 and 5) of the same section, made before and after staining with silver nitrate.

Evidently these are not merely deposits of urine salts in the lumen of the tubules, which would be solid cylinders and not hollow, as these undoubtedly are. The lumps, by their shape and frequently by a kind of nucleus, as also by their position against the membrana propria and their arrangement in a circle, betray a close relation to epithelial cells. Therefore the conclusion is

justified that they are indeed epithelial cells impregnated with calcium.

EXPLANATION OF PLATE 1.

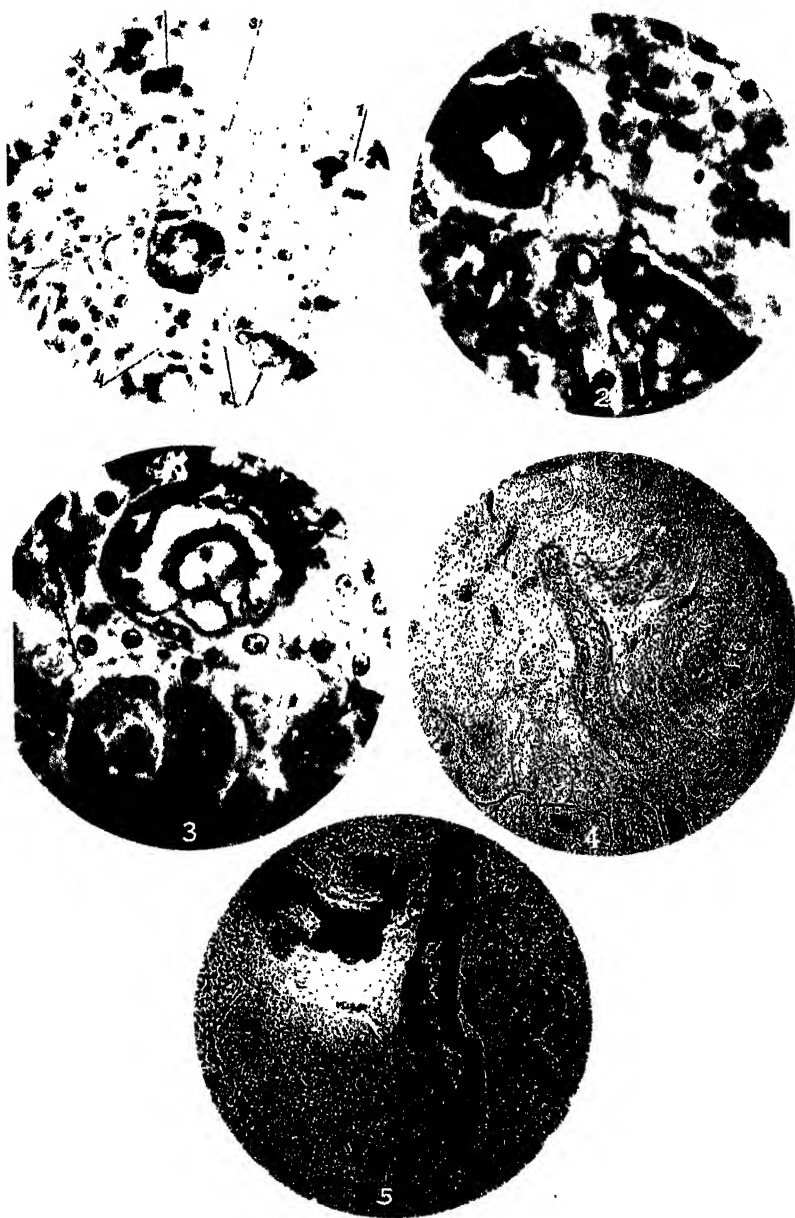
FIG. 1. Section of the kidney of a rat suffering from vitamin A deficiency. Stained with hematoxylin (Delafeld)-eosin. Zeiss comp. oc. 4., obj. E. 1, calcified epithelial cells (square and irregular shapes). 2, calcified epithelial cells (round and oval). 3, calcified tubules. The one on the left shows the first signs of calcification; remains of nuclei are still visible in it. 4, calcified nuclei arranged in a circle.

FIG. 2. Part of kidney shown in Fig. 1. Zeiss homal 4, obj. E. Some very dark nuclei are visible. The oval lumps are clearly visible, but show no particular structure.

FIG. 3. Kidney. Hematoxylin (Delafeld)-eosin. Zeiss homal 4, obj. E. A calcified tubule. In some lumps there is still something visible which resembles a nucleus. The other tubules are normal.

FIG. 4. Kidney, not stained. Zeiss comp. oc. 4., obj. E. In the center are two calcified loops. The structure is no longer normal, yet some nuclei can still be recognized.

FIG. 5. The same section as in Fig. 4, after being stained with silver nitrate. Consequently the structure cannot be recognized. The calcium casts prove to be hollow.



(van Leersum: Vitamin A deficiency.)

THE CONCENTRATION OF VITAMIN B.

IV. ON THE CONCENTRATION AND THE SEPARATION OF THE TWO COMPONENTS OF VITAMIN B.

By P. A. LEVENE.

(From the Laboratories of The Rockefeller Institute for Medical Research,
New York.)

(Received for publication, July 11, 1928.)

In 1926 Levene and van der Hoeven¹ communicated a method of concentration of vitamin B which consisted of two steps: first, deamination of the starting material; second, adsorption of the material on silica gel. In the early experiments of Levene and van der Hoeven daily doses of 0.1 mg. of the material sufficed to maintain the normal growth of white rats. In the majority of experiments the period of observation was very short and only in one did it last 14 days. However, when the observations were extended to longer periods, the material was found ineffective after the 1st week. Since then it has been found through the important contributions of Goldberger,² of Chick,³ of Peters,⁴ of Sherman,⁵ and of others that vitamin B consists of at least two components, one heat-stable, the antipellagra factor, and the other heat-unstable, the antineuritic factor.

New tests on the material prepared by the method described in the article of Levene and van der Hoeven have shown that it contains the heat-unstable factor B₁ of the English Accessory Food Factors Committee, or vitamin F of H. C. Sherman, in high

¹ Levene, P. A., and van der Hoeven, B. J. C., *J. Pharmacol. and Exp. Therap.*, 1926, xxix, 227.

² Goldberger, J., Wheeler, G. A., Lillie, R. D., and Rogers, L. M., *Pub. Health Rep., U. S. P. H. S.*, 1926, xli, 297.

³ Chick, H., and Roscoe, M. H., *Biochem. J.*, 1927, xxi, 698.

⁴ Peters, R. A., *Biochem. J.*, 1924, xviii, 858. Kinnersley, H. W., and Peters, R. A., *Biochem. J.*, 1925, xix, 820; 1928, xxii, 419.

⁵ Sherman, H. C., and Axtmayer, J. H., *J. Biol. Chem.*, 1927, lxxv, 207.

concentration. A daily dose of 0.07 mg. added to a daily dose of the heat-stable factor, namely to 300 mg. of heated dried yeast, or to 30.0 mg. of heated material referred to as (O.W.) fraction, sufficed to maintain normal growth.

It was further found that silica gel has the power of adsorbing both factors, but that it adsorbs the heat-unstable fraction preferentially. Thus a unit weight of dried yeast contains 1 part of the heat-stable factor to $7\frac{1}{2}$ parts of the heat-unstable, whereas a unit weight of the material obtained by elution from the silica contains 1 part of the first to 30 of the second.

Thus by means of silica gel it is possible to obtain:

1. A fraction containing only the heat-stable factor.
2. A fraction containing the two factors in high concentration (Fraction 159). Daily doses of 2.2 mg. of this material in its best samples are capable of maintaining normal growth of white rats and 0.07 mg. of the same material contains an effective daily dose of the heat-unstable component. Thus the ratio is 1:30.
3. By means of nitrous acid the activity of the heat-stable factor is destroyed, whereas that of the heat-unstable remains intact. Hence, by deaminizing the starting material, (O.W.) fraction, and subsequently extracting it with silica, a material is obtained which contains only the heat-unstable factor (Fraction 349). Daily doses of 0.07 mg. of this material suffice to maintain the normal growth of white rats.

EXPERIMENTAL.

Preparation of Starting Material.—The starting material was of the same nature as the concentrate of Osborne and Wakeman. The procedure for its preparation, however, differed essentially from that of Osborne and Wakeman.

50 pounds of brewers' yeast (not compressed), taken up in 5 gallons of 95 per cent alcohol, were warmed for 4 hours at 60° and then filtered. The filtrate was concentrated under reduced pressure to a volume of 1 liter. An equal volume of 95 per cent alcohol was then added. The precipitate which was thus formed was removed by centrifugalization and to the decanted clear, supernatant liquid enough 98.5 per cent alcohol was added to make the concentration of alcohol equal to 80 per cent. A precipitate was formed which was washed with absolute alcohol and

then shaken mechanically with acetone, the acetone being renewed so long as it continued to extract. The final material, which was capable of maintaining normal growth in doses of 30 mg. per day, will be referred to as (O.W.) fraction.

Fractionation by Means of Silica Gel.—50 gm. of the starting material, (O.W.) fraction, were taken up in 500 cc. of water and a small portion remaining undissolved was removed by centrifugalization. The supernatant liquid was decanted and diluted with water to a volume of 4 liters. The solution was then acidulated to pH 3.0 by means of hydriodic acid. To this solution 2 kilos of silica gel were added and the mixture was agitated for 15 minutes. The filtrate was reextracted with 1 kilo of silica gel and the operation was repeated four times (the total number of extractions being six). The final filtrate from the silica gel was neutralized with lithium hydroxide and concentrated to 100 cc. The concentrate was allowed to stand overnight in the refrigerator at 0° to allow a sediment to settle. It was then centrifugalized and to the supernatant liquid alcohol (98.5 per cent) was added to a concentration of 80 per cent alcohol. The precipitate thus formed was washed repeatedly by mechanical shaking with alcohol and then dried under reduced pressure. The yield of this material was about 28 to 30 gm. out of the original 50 gm. This material was free from B₁, but 30 mg. of it on addition of the B₁ factor were capable of maintaining normal growth of white rats.

Fraction Adsorbed on Silica Gel.—The material adsorbed on the silica was liberated in the following way. The first portion (2 kilos) of gel was suspended in 4 liters of water containing sufficient hydriodic acid to bring the pH to 3.0 and the silica was then filtered and washed with water on the suction funnel. It was then suspended in 6 liters of water and the mixture, brought to pH 9.8 by means of lithium hydroxide, was agitated for about 15 minutes after the required hydrogen ion concentration had been attained. The liquid was removed by filtration and the silica was washed with water until the wash waters became colorless. All filtrates were combined, neutralized with hydriodic acid, and concentrated to 75 cc. To this solution 50 cc. of alcohol were added and the mixture was allowed to settle overnight. The mixture was then centrifugalized. The supernatant liquid was concentrated to 40 cc. To this solution 40 cc. of alcohol were

added and the mixture was allowed to stand overnight. The precipitate was then removed by centrifugalization and the supernatant liquid was slowly poured into 1000 cc. of acetone. A precipitate was formed which was washed with acetone and dried. The yield of this material was between 6 and 7 gm. and the material generally contained about 30 per cent of mineral matter. The subsequent portions of silica gel were treated in the same way. The combined material obtained in this manner contained all the heat-unstable factor, factor B₁ of the English workers or vitamin F of Sherman, and part of the heat-stable factor in a concentrated state.

Properties of Material Containing Factor B₁.—This material was obtained in the form of a slightly yellow powder, completely soluble in water and partly soluble in ethyl and in methyl alcohols. The substance gave negative biuret and Millon tests. It did not reduce Fehling's solution, but did so after hydrolysis. When a small sample was heated with strong alkali, hydrogen sulfide, demonstrable by means of lead acetate, was evolved, but the nitroprusside test was very feeble and transitory. Under the same conditions a strong odor of indole or skatole was developed. The substance had the following composition.

C 31.54, H 4.99, N 8.92, NH₂ 1.9, $\frac{\text{amino N}}{\text{total N}}$ 22.02, P 8.92.

The activity of the material varied. The best samples of this fraction were capable of maintaining normal growth of white rats fed on the standard vitamin B-free diet in daily doses of 2.2 mg. (calculated as ash-free material). The length of experiments varied from 18 to 24 days. Continued half normal growth was maintained with daily doses of 0.7 mg. Thus, this material contained all the factors of vitamin B. The heat-unstable fraction, however, predominated in it, for 0.07 mg. of the material sufficed to maintain normal growth when 30.0 mg. of (O.W.) fraction heated to 140° were added to the diet. When the daily dose of this fraction was raised to 0.7 mg., that of the heated material remaining unchanged, the growth of the animals exceeded normal growth by 30 per cent.

After it became known that vitamin B is composed of at least two factors, the question arose as to the relative concentrations of

each factor in this material. In order to solve this problem it was necessary to establish their relative proportions in the yeast itself. This problem is now accessible to solution, inasmuch as one of the fractions can be destroyed by heat. Daily doses of 300 mg. of the acetone-dried yeast employed by us were required to maintain the normal growth of white rats under our conditions of experiment. When the yeast was heated at 135° it became ineffective in preventing loss of weight of the white rats. However, when to 300 mg. of

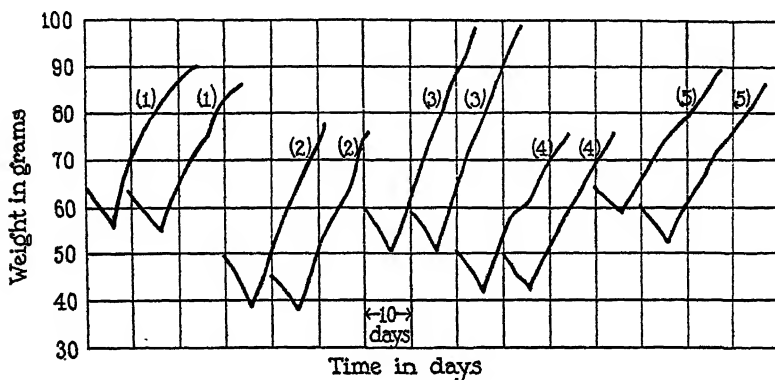


FIG. 1. In the curves given the daily dose was: for Curves 1, 3.0 mg. of Fraction 159; Curves 2, 30.0 mg. of Fraction 247, 1.0 mg. of Fraction 159; Curves 3, 30.0 mg. of Fraction 247, 50.0 mg. of acetone-dried yeast; Curves 4, 30.0 mg. of Fraction 247, 0.07 mg. of Fraction 159; Curves 5, 30.0 mg. of Fraction 247, 0.1 mg. of Fraction 349.

Fraction 159 = material obtained by elution from silica. Fraction 247 = heated (O.W.) fraction. Fraction 349 = deaminized (O.W.) fraction extracted with silica.

heated yeast 50 mg. of the acetone-dried yeast were added, the growth of the white rats exceeded somewhat the normal. Thus it is warranted to assume that 40 mg. of the dried yeast may be regarded as the quantity containing 1 unit of the heat-unstable component and 300 mg. as the quantity containing 1 unit of heat-stable component. Hence 300 mg. of yeast, containing 1 unit of the heat-stable component, contain 7.5 units of the heat-unstable substance, or the ratio of the first to the second is 1:7.5. In order to test the relative concentrations of each of the components

in our material it was necessary to establish the minimum weight of the material per day required to maintain normal growth when added to the daily dose of the heat-stable material. It was found that 300 mg. of heated dried yeast or 30 mg. of the heated (O.W.) fraction (No. 247) contained the daily requirement of heat-stable component and it was further found that 0.07 mg. of the best samples of Fraction 159 added to the 30 mg. of heat-stable material sufficed to maintain normal growth. Thus a unit weight of Fraction 159 (approximately 2.2 mg.) contains $30 \left(\frac{2.2}{0.07} \right)$ units of the heat-labile substance. Thus the ratio is 1:30, a fact which shows that silica gel has a greater power of adsorption for the heat-labile component than for the heat-stable.

Preparation of Heat-Labile Component (B_1) Free from B_2 .

The preparation of this material was accomplished by deaminizing the starting material with nitrous acid and treating the material prepared in this manner by the method described in the article of Levene and van der Hoeven. If the deamination was carried out rigorously, the material did not contain any detectable quantities of the heat-stable material. The best samples obtained in this manner were capable of maintaining normal growth in daily doses of 0.07 to 0.1 mg.

ACETYL MONOSES.

V. THE RATES OF HYDROLYSIS OF TETRAACETYLMETHYLMAN- NOSIDES AND OF TRIACETYLMETHYLLYXOSIDES.

BY P. A. LEVENE AND M. L. WOLFROM.*

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(Received for publication, July 7, 1928.)

Fischer, Bergmann, and Rabe¹ made the important discovery that on methylation of bromotriacetyl-*l*-rhamnose two crystalline triacetylmethyl-*l*-rhamnosides were formed, one with the specific rotation of $+45.7^\circ$ in acetylene tetrachloride, the other with the specific rotation of $+28.0^\circ$ in the same solvent. The latter possessed an unusual stability of one of the acetyl groups, so that on saponification with alkali a monoacetylmethyl-*l*-rhamnoside was obtained. Dale² and later Levene and Sobotka³ have shown that bromotetraacetyl-*d*-mannose also yields a tetraacetylmethyl-*d*-mannoside which on saponification with alkali yields a monoacetylmethyl-*d*-mannoside. This form will be referred to in this discussion as the third form. The form of the tetraacetylmethyl-*l*-rhamnoside with the rotation of $+45.7^\circ$ will be referred to as the second form, and the first will be considered the form which is obtained on acetylation of α -methylrhamnoside. Fischer, Bergmann, and Rabe regarded the second form as the β form of the first α form. The basis for their conclusion was the fact that the first and the second forms showed approximately the same rate of hydrolysis with acid, whereas the third form hydrolyzed at a much higher velocity.

From bromotetraacetylmannose likewise two crystalline methyl-

* National Research Fellow in Chemistry.

¹ Fischer, E., Bergmann, M., and Rabe, A., *Ber. chem. Ges.*, 1920, liii, 2362.

² Dale, J. K., *J. Am. Chem. Soc.*, 1924, xlii, 1046.

³ Levene, P. A., and Sobotka, H., *J. Biol. Chem.*, 1926, lxxvii, 759, 771.

tetraacetates were obtained, one corresponding to the second form, the other to the third form of rhamnose. To the former Hudson⁴ is inclined to attribute the $\langle 1,4 \rangle$ lactal structure. We were successful this year in preparing a small quantity of the second form of triacetylmethylmannoside with the specific rotation of -50.0° . Thus, we were in possession of three forms of tetraacetylmethylmannoside and two forms of triacetylmethyllyxoside. It was thought that a comparison of the rates of hydrolysis of all these forms might aid in their classification. Irvine and Burt⁵ have shown that the so called γ form of methylmannoside hydrolyzed at a higher rate than the common form and Levene and Meyer⁶ have shown that the γ form had the $\langle 1,4 \rangle$ lactal structure. Hence a comparison of the rates of hydrolysis of the first and of the second forms of tetraacetylmethylmannoside should give an indication as to the lactal structures of the two forms. It has now been found that they both hydrolyze under similar conditions with approximately the same rates and that therefore it is warranted to assume that the second form possesses the $\langle 1,5 \rangle$ and not the $\langle 1,4 \rangle$ structure inasmuch as the latter should have hydrolyzed with a much higher velocity.

The rates of hydrolysis of the third forms of mannose and of lyxose were of the same order of magnitude. In this respect their behavior is similar to that of the third form of triacetylmethylrhamnoside of Fischer, Bergmann, and Rabe.¹ To this third form these authors, as well as Hudson,⁴ assigned the $\langle 1,3 \rangle$ structure. However, the possibility is not excluded that they possess the $\langle 1,4 \rangle$ lactal structure. Work on this phase of the problem is now in progress.

EXPERIMENTAL.

All substances used were carefully purified. The specific rotations in chloroform solution of the tetraacetylmethyl-*d*-mannosides used were: α , $+49^\circ$; β , -45° ; γ , -26° . The β form was isolated in low yield directly from some of the crude preparations obtained by treating syrupy acetobromomannose with methyl alcohol and

⁴ Hudson, C. S., *J. Am. Chem. Soc.*, 1926, *xlvi*, 1424, 1434.

⁵ Irvine, J. C., and Burt, W., *J. Chem. Soc.*, 1924, *cxv*, 1343.

⁶ Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, 1928, *lxxvi*, 809.

⁷ Nomenclature of E. Fischer.

silver carbonate. It was separated from the γ form by dissolving the crude material in a small amount of chloroform and adding ether, the β form being nearly insoluble in the latter solvent. The α -methyl-*d*-lyxoside triacetate used showed a specific rotation of

TABLE I.

Hydrolysis of Acetylated Methyl-d-Mannosides.

Solvent, 0.1 N HCl in 50 per cent ethyl alcohol. Hydrolyzed 90 minutes at 98°.

$l = 2$ dm.

$\lambda = 5892 \text{ \AA.}$

Form.	c (Mannoside.)	α_D^{25}	$[\alpha]_D^{25}$	Reduction.
		<i>degrees</i>	<i>degrees</i>	
α	2.72	+3.80	+70	Slight.
	1.13	+1.52	+67	"
	1.12	+1.58	+71	"
β	1.12	-1.24	-55	"
	1.13	-1.20	-53	"
γ	1.13	+0.38	+17	Heavy.
	2.71	+0.86	+16	"

TABLE II.

Hydrolysis of Acetylated Methyl-d-Lyxosides.

Solvent, 0.01 N HCl in 50 per cent ethyl alcohol. Hydrolyzed 90 minutes at 98°.

$l = 2$ dm.

$\lambda = 5892 \text{ \AA.}$

Form.	c (Lyxoside.)	α_D^{25}	$[\alpha]_D^{25}$	Reduction.
		<i>degrees</i>	<i>degrees</i>	
α	2.85	+2.57	+45	Very slight.
	2.87	+2.66	+46	" "
γ	2.86	-0.95	-17	Heavy.
	2.86	-0.88	-15	"
	1.20	-0.41	-17	"

+30° in chloroform solution. The specific rotation of the γ form in chloroform solution was -104°.

The hydrolysis procedure adopted was to dissolve the acetate in a measured amount of absolute alcohol equal to half the total

volume used (5.00 or 10.00 cc.). The solution was then brought to exact total volume with an aqueous solution of hydrochloric acid of twice the final strength desired. All the solutions were sealed in glass tubes and heated at 98° for 90 minutes, after which the tubes were opened and polarimetric readings and reduction tests taken. The data obtained are recorded in Tables I and II. These tables show that the γ forms were hydrolyzed to the sugars, as the final specific rotations obtained were approximately those of the free sugars ($+15^{\circ}$ for mannose and -14° for lyxose in water solution). The final rotations of the α and β forms indicate that little hydrolysis was effected in these cases.

CONFIGURATIONAL RELATIONSHIPS OF METHYLBUTYL
CARBINOL AND OF 2-HYDROXYCAPROIC ACID
TO LACTIC ACID.

WITH A NOTE ON THE RELATIONSHIP OF CHEMICAL STRUCTURE
TO OPTICAL ACTIVITY.

By P. A. LEVENE AND H. L. HALLER.

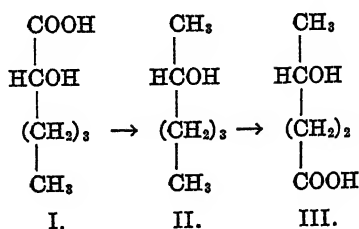
(From the Laboratories of The Rockefeller Institute for Medical Research,
New York.)

(Received for publication, July 7, 1928.)

In the earlier papers¹ of this series the configurational relationships have been established between a series of hydroxy acids and the reference substance, lactic acid, and also the configurational relationships of a series of secondary carbinols to the same reference substance. In fact, it was found that the knowledge of the configurations of the hydroxy acids served to elucidate the configurations of the carbinols and *vice versa*. As the work progressed it became evident that the data obtained through it may lead to the deduction of some more comprehensive rules regarding the relationship between chemical structure and optical rotation, as will be shown later in this paper in the tabulation of the data thus far obtained.

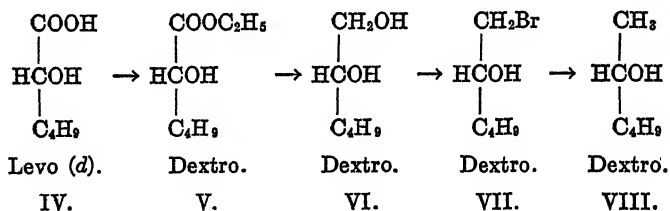
The present communication deals with the relationship of 2-hydroxycaproic acid and of methylbutyl carbinol (hexanol-(2)) to lactic acid. The relationship was established readily because of the knowledge of the configuration of 4-hydroxyvaleric acid to lactic acid. The process of reasoning which led to the conclusion of the above relationship is readily seen when only the three significant substances are considered.

¹ Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, 1925, lxxv, 49; 1926, lxxvii, 329; 1926, lxix, 165, 569; 1927, lxxiv, 343; 1928, lxxvi, 415; 1928, lxxvii, 555. Levene, P. A., Walti, A., and Haller, H. L., *J. Biol. Chem.*, 1926-27, lxxi, 465. Levene, P. A., Haller, H. L., and Walti, A., *J. Biol. Chem.*, 1927, lxxii, 591.



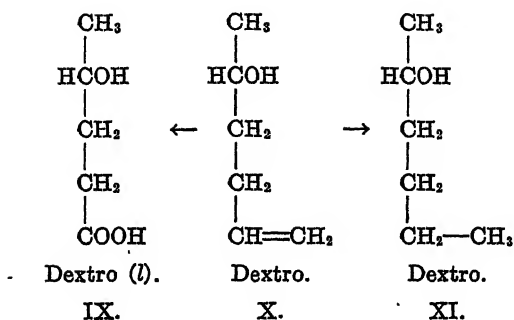
Of the three substances the configuration of only one (III) is known and in order to correlate the configurations of the three it suffices to correlate each of the two acids with the carbinol of formula (II).

The process of correlating (I) to (II) was the same as that employed on other occasions in this series of investigations and is given by the following set of reactions.



It must be mentioned, however, that, as on previous occasions, the glycol employed in the preparation was not that prepared by the reduction of the corresponding ester, but that from hydroxymethylbutyl ketone reduced asymmetrically by yeast. From the ester enough glycol was obtained to determine the direction of its rotation. The glycol was further identified by means of its α -naphthyl-diurethane.

The second phase of the problem consisted of the correlation of the configuration of the carbinol II (the same as VIII) with that of 4-hydroxyvaleric acid III (the same as IX). This task was accomplished by the following set of reactions.

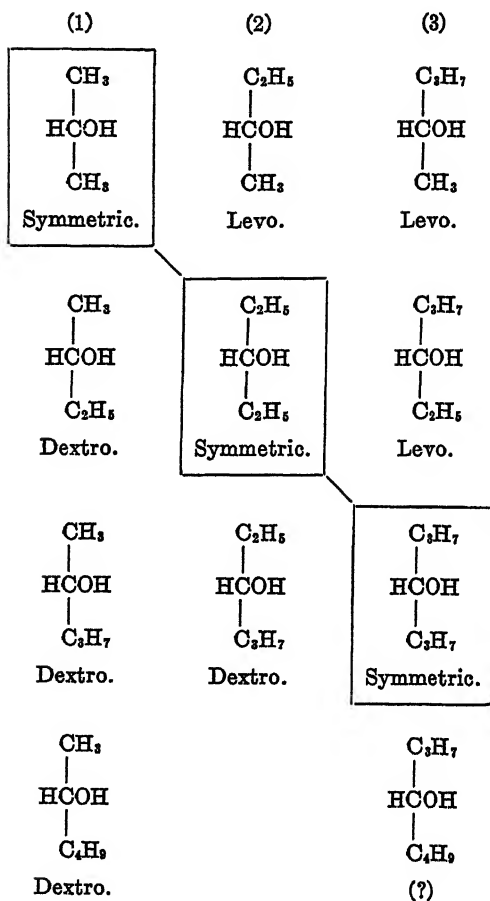


As was to be expected, dextro-methylbutyl carbinol (hexanol-(2)) can be derived either from levo-2-hydroxycaproic acid (d) or from dextro-4-hydroxyvaleric acid (l).

It was also observed that in the case of levo-2-hydroxycaproic acid, of the d series, the direction of rotation on passing from the undissociated acid to the ion changes to the right.

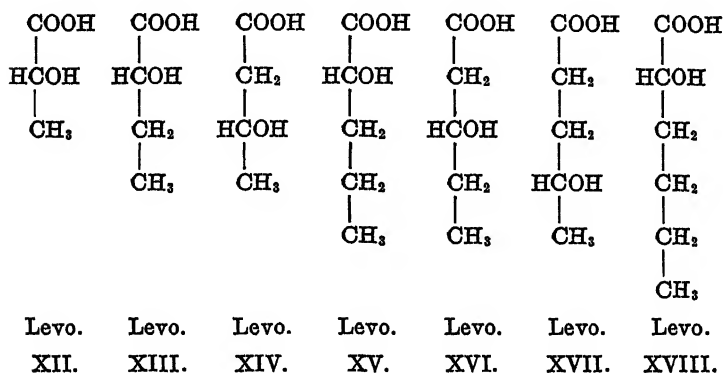
The diagram on p. 478 contains a summary of the results of the observations on the carbinols. Of these three series of carbinols the configurations are known of all save the last member of Column 3, and we venture to predict that this carbinol will be dextrorotatory. In Column 1, all members following the first member, which is symmetric, are dextrorotatory. In Column 2, the carbinol above the symmetric is levorotatory and that below is dextrorotatory. In Column 3 those above the symmetric are levorotatory and the one below we expect to be dextrorotatory. The reason for our belief lies in the fact that one property connects all the carbinols above the symmetric and another all those below the symmetric; namely, that in the former the radicle with the heavier mass is located at the top, in the latter at the bottom, of the figures given in the chart. Thus in this group of substances the direction of rotation seems to be determined by the distribution of the masses around the asymmetric carbon atom; in other words, the law enunciated by Guye² holds for this simplest group of optically active substances. In this group of substances only one of the four radicles attached to

² Guye, P. A., *Compt. rend. Acad.*, 1890, cx, 714; 1893, cxvi, 1378, 1451.

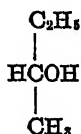


the asymmetric carbon atom is of distinctly different polarity from the remaining three.

A different aspect of the question is presented by the scrutiny of the directions of rotation of the hydroxy acids shown below.

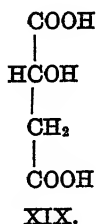


This group of substances differs from the carbinols in that in place of a simple alkyl group, a polar carboxyl group is introduced. It is also noticed that in all the acids up to (XVIII) the group of higher polarity is also the one of greater mass and, if mass is the determining factor, these acids should rotate in the same direction as



that is, they should rotate to the left; this condition was found to hold experimentally. However, the behavior of the acid (XVIII) contradicts this assumption. In this acid the heavier group is the non-polar and if mass were the determining factor, the acid should rotate to the right. Thus, it would appear that when a non-polar group is replaced by a strongly polar group the effect of polarity seems to outweigh the effect of mass and that the position of the polar group determines the direction of rotation. In this

connection it is of interest to note that the malic acid of the configuration given in the figure



again is dextrorotatory. In this case the polarities of the two significant groups are of about the same character and the determining factor is the weight of the respective groups. It will be important to extend the series of observations to acids in which the weight of the alkyl group is much greater than that of the butyl radicle. Possibly a point will be reached when the effect of mass will outweigh the effect of polarity.

It is our intention to continue the investigation on the relationship of chemical structure to optical activity from the view-point discussed in this paper.

EXPERIMENTAL.

Relationship of 2-Hydroxy-n-Caproic Acid to 1,2-Dihydroxyhexane.

Levo-2-Hydroxy-n-Caproic Acid.—The inactive acid was obtained from 2-bromo-*n*-caproic acid. 122 gm. of 2-bromo-*n*-caproic acid were added to 600 cc. of water containing 86.5 gm. of potassium carbonate. The solution was heated under a reflux condenser on a boiling water bath for 8 hours. It was then cooled, a solution of 64 gm. of concentrated sulfuric acid in 100 cc. of water was added slowly, and the solution was extracted with ether in a continuous ether extractor. The ether extract was dried over sodium sulfate and the ether removed under reduced pressure. The acid was dissolved in chloroform and neutralized with 1 equivalent of cinchonidine. Resolution was effected by the procedure described by Levene, Mori, and Mikeska.³ The cinchonidine salt

³ Levene, P. A., Mori, T., and Mikeska, L. A., *J. Biol. Chem.*, 1927, **lxxv**, 337.

was decomposed in the usual way and the acid converted to the sodium salt. In water it had the following rotation.

$$[\alpha]_D^{20} = \frac{+ 3.15^\circ \times 100}{2 \times 13.3} = + 11.8^\circ.$$

To 3 cc. of the above solution were added 1.2 cc. of 2.43 N hydrochloric acid. The rotation was observed immediately. For the free acid,

$$[\alpha]_D^{20} = \frac{- 0.15^\circ \times 100}{2 \times 8.1} = - 1.9^\circ.$$

Dextro-Ethyl-2-Hydroxy-n-Caproate.—A solution of 13 gm. of concentrated sulfuric acid in 50 cc. of absolute alcohol was added slowly to 34 gm. of thoroughly dried sodium 2-hydroxy-*n*-caproate ($[\alpha]_D^{20} = +11.8^\circ$) suspended in 150 cc. of absolute alcohol. The mixture was agitated vigorously with a mechanical stirrer during the addition of the acid solution. It was then refluxed for 7 hours and allowed to stand overnight. Potassium carbonate was added to neutralize the excess acid, the salts were precipitated with dry ether, and the solution was filtered. After being dried over anhydrous sodium sulfate the solvent was removed and the ester distilled under reduced pressure. It distilled at 91–93°, *p* = 17 mm. The yield was 28 gm. It analyzed as follows:

5.260 mg. substance: 11.600 mg. CO₂ and 4.600 mg. H₂O.

C₈H₁₆O₃. Calculated. C 60.00, H 10.00.

Found. " 60.13, " 9.78.

In a 2 dm. tube the optical rotation without solvent was $\alpha_D^{20} = +11.0^\circ$.

Dextro-1,2-Dihydroxyhexane.—Dextro-ethyl-2-hydroxy-*n*-caproate ($\alpha_D^{20} = +11.0^\circ$ in a 2 dm. tube) was reduced with sodium and glacial acetic acid in the apparatus described by Levene and Allen.⁴ The procedure for the reduction was the same as that previously

⁴ Levene, P. A., and Allen, C. H., *J. Biol. Chem.*, 1916, xxvii, 443.

described for the reduction of other hydroxy acids.⁵ The glycol analyzed as follows:

5.175 mg. substance: 11.980 mg. CO₂ and 5.085 mg. H₂O.
 C₈H₁₄O₂. Calculated. C 61.02, H 11.95.
 Found. " 63.11, " 10.99.

In absolute alcohol it had the following rotation.

$$[\alpha]_D^{20} = \frac{+ 1.95^\circ \times 100}{2 \times 9.0} = + 10.8^\circ.$$

Di-(α-Naphthylurethane) of Dextro-1,2-Dihydroxyhexane (Hexylene-Bis-α-Naphthyl Carbamate).—The glycol obtained in the above experiment was converted into its di-(naphthylurethane) in the usual manner. It was recrystallized from absolute alcohol. It gradually softened and melted at 155–160° and analyzed as follows:

0.1000 gm. substance: 4.20 cc. 0.1 N HCl (Kjeldahl).
 C₂₈H₂₈O₄N₂. Calculated. N 6.14. Found. N 5.88.

In glacial acetic acid it had the following rotation.

$$[\alpha]_D^{24} = \frac{- 0.50^\circ \times 100}{2 \times 4.4} = - 5.7^\circ.$$

Preparation of Dextro-1,2-Dihydroxyhexane from d,l-1-Chloro-2-Hydroxyhexane.

d,l-1-Chloro-2-Hydroxyhexane.—This substance was obtained on condensation of chloroacetaldehyde with butyl magnesium bromide. The procedure for the preparation was the same as that described in the preparation of 1-chloro-2-hydroxypentane.⁶ The chlorohydrin distilled at 74–77°, p = 12 mm. It analyzed as follows:

0.1117 gm. substance: 0.2162 gm. CO₂ and 0.0962 gm. H₂O.
 0.1126 " " : 0.1136 " AgCl.
 C₆H₁₃OCl. Calculated. C 52.75, H 9.52, Cl 26.00.
 Found. " 52.78, " 9.63, " 24.88.

⁵ Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, 1926, lxi, 165. Levene, P. A., Haller, H. L., and Walti, A., *J. Biol. Chem.*, 1927, lxxii, 593.

⁶ Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, 1928, lxxvii, 555.

Chloromethylbutyl Ketone (1-Chlorohexanone-(2)).—The chlorohexanol obtained as described above was oxidized with potassium dichromate and sulfuric acid. To 50 gm. of 1-chloro-2-hydroxyhexane were added 40 gm. of finely pulverized potassium dichromate. This mixture, cooled in an ice water bath, was vigorously stirred with a mechanical stirrer. A solution of 60 gm. of concentrated sulfuric acid in 75 cc. of water was then slowly dropped into the mixture. After the addition of all the acid, stirring was continued for 2 hours and the reaction mixture was allowed to come to room temperature. It was then diluted with water and extracted with ether. The ether extract was dried over anhydrous sodium sulfate. The ether was removed and the chloroketone distilled. It boiled at 70° , $p = 15$ mm. It analyzed as follows:

5.905 mg. substance: 11.615 mg. CO_2 and 4.450 mg. H_2O .
 $\text{C}_6\text{H}_{11}\text{OCl}$. Calculated. C 53.53, H 8.25.
Found. " 53.63, " 8.43.

Hydroxymethylbutyl Ketone (Hexanol-(1)-one-(2)).—A mixture of 100 gm. of chloromethylbutyl ketone, 125 gm. of dried potassium formate, and 150 cc. of dry methyl alcohol was heated under a reflux condenser on a boiling water bath overnight. The reaction mixture was cooled, dry ether was added, and the solution was filtered. After being dried over anhydrous sodium sulfate, the solvent was removed and the hydroxyketone was distilled under reduced pressure. The fraction which distilled at $83\text{--}85^{\circ}$, $p = 15$ mm., was collected and employed for the reduction to the glycol.

Dextro-1,2-Dihydroxyhexane.—A solution of 50 gm. of freshly distilled hydroxymethylbutyl ketone in 50 cc. of absolute alcohol was slowly dropped into an actively fermenting mixture of 5000 cc. of water, 500 gm. of sugar, and 500 gm. of yeast. About 100 gm. of yeast were added every day for 5 days and the fermentation mixture was frequently shaken. On the 7th day the mixture was filtered and worked up in the usual way.⁷ On redistillation the glycol boiled at $110\text{--}113^{\circ}$, $p = 6$ mm. It analyzed as follows:

5.380 mg. substance: 11.985 mg. CO_2 and 5.735 mg. H_2O .
 $\text{C}_6\text{H}_{14}\text{O}_2$. Calculated. C 61.02, H 11.95.
Found. " 60.74, " 11.93.

⁷ Neuberg, C., and Korb, E., *Biochem. Z.*, 1918, xcii, 96. Farber, E., Nord, F. F., and Neuberg, C., *Biochem. Z.*, 1920, cxii, 313.

In absolute alcohol it had the following rotation.

$$[\alpha]_D^{22} = \frac{+ 2.00^\circ \times 100}{1 \times 13.14} = + 15.2^\circ.$$

Di-(α -Naphthylurethane) of Dextro-1,2-Dihydroxyhexane (Hexylene-Bis- α -Naphthyl Carbamate).—1 part of glycol obtained as described above and 2.8 parts of α -naphthyl isocyanate were heated on the steam bath for 15 minutes. The reaction product soon crystallized. It was dissolved in boiling absolute alcohol, filtered, and allowed to crystallize. It melted at 172–174° and analyzed as follows:

0.1000 gm. substance required 4.30 cc. 0.1 N HCl.

$C_{28}H_{28}O_4N_2$. Calculated. N 6.14. Found. N 6.02.

In glacial acetic acid it had the following rotation.

$$[\alpha]_D^{23} = \frac{- 0.50^\circ \times 100}{2 \times 2.48} = - 10.1^\circ.$$

Conversion of Dextro-1,2-Dihydroxyhexane into Dextro-Methylbutyl Carbinol.

Dextro-1-Bromo-2-Hydroxyhexane.—Into 17.5 gm. of 1,2-dihydroxyhexane ($[\alpha]_D^{22} = +15.2^\circ$), cooled in an ice water bath, were passed 14.5 gm. of dry hydrogen bromide. The reaction mixture was heated on the steam bath for $\frac{1}{2}$ hour, cooled, ice and chloroform added, and neutralized with solid potassium carbonate. The chloroform solution was dried over sodium sulfate. After removal of the chloroform the bromohydrin was distilled. It boiled at 93–95°, $p = 17$ mm. It analyzed as follows:

0.1218 gm. substance: 0.1240 gm. AgBr.

$C_6H_{13}OBr$. Calculated. Br 44.2. Found. Br 43.3.

In a 1 dm. tube without solvent it had the rotation $\alpha_D^{22} = +2.85$.

Dextro-Methylbutyl Carbinol.—The bromohydrin obtained in the above experiment was reduced in alkaline solution with hydrogen and colloidal palladium. The procedure was the same as that previously described for the reduction of 1-iodo-3-hydroxybutane.⁸

⁸ Levene, P. A., Walti, A., and Haller, H. L., *J. Biol. Chem.*, 1926–27, lxxi, 467.

The ether extract was dried over anhydrous sodium sulfate and the ether distilled. The carbinol was then fractionally distilled and a fraction which boiled at 135–140° was collected. In ether it had the following rotation.

$$[\alpha]_D^{24} = \frac{+ 1.55^\circ \times 100}{1 \times 10.36} = + 15.0^\circ.$$

It was further identified by converting it into its α -naphthylurethane.

α -Naphthylurethane of Dextro-Methylbutyl Carbinol (Hexyl- α -Naphthyl Carbamate).—1 part of carbinol obtained in the above experiment and 1.7 parts of α -naphthyl isocyanate were heated on the steam bath for 15 minutes. On standing overnight it crystallized. It was recrystallized several times from dilute alcohol. It melted at 61–65° and analyzed as follows:

0.1000 gm. substance required 3.98 cc. 0.1 N HCl.

$C_{17}H_{21}O_2N$. Calculated. N 5.20. Found. N 5.57.

In absolute alcohol it had the following rotation.

$$[\alpha]_D^{24} = \frac{+ 1.00^\circ \times 100}{2 \times 8.0} = + 6.3^\circ.$$

Relationship between Dextro-Methylbutyl Carbinol and Dextro-4-Hydroxyvaleric Acid.

Δ^5 -Hexenol-(2).—This carbinol was obtained on reduction of allylacetone with sodium and glacial acetic acid. The allylacetone was prepared by the condensation of allyl bromide and acetoacetic ester, followed by ketone decomposition. It was reduced in the apparatus described by Levene and Allen.⁴ The procedure was the following.

10.5 gm. of sodium were emulsified in 75 cc. of toluene. A solution of 15 gm. of allylacetone dissolved in 20 cc. of glacial acetic acid was then introduced at such a rate that the addition required 10 minutes. During the course of the reaction, 50 cc. of toluene were added gradually through the condenser. After the addition of all the ketone, 20 cc. of glacial acetic acid were added and after refluxing had ceased, absolute alcohol was introduced until all the sodium had reacted. The reaction mixture was

cooled, and dilute alcohol was added, followed by water. The mixture was then steam-distilled and the chloroform solution of the carbinol separated from the aqueous layer. After being dried over potassium carbonate, the chloroform solution was fractionally distilled. The carbinol boiled at 138–140°.

Dextro- Δ^5 -Hexenol-(2).—To 100 gm. of inactive Δ^5 -hexenol-(2) dissolved in 200 cc. of dry pyridine were added 148 gm. of phthalic anhydride. The mixture was allowed to stand overnight and then heated on the steam bath for 1 hour. The solution was cooled, ice and chloroform were added, followed by an excess of concentrated hydrochloric acid. The acid ester was extracted with chloroform, the extract was washed with water and dried over sodium sulfate. The chloroform was removed under reduced pressure and the residue was dissolved in a slight excess of sodium carbonate. The solution was then extracted with ether to remove any unchanged carbinol. It was then acidified with hydrochloric acid and the acid ester extracted with chloroform. The chloroform extract was washed with water and dried over anhydrous sodium sulfate. After removal of most of the chloroform the residue was poured into petroleum ether, from which the phthalate readily crystallized.

The phthalate was dissolved in acetone and converted into the brucine salt. This salt was recrystallized several times from acetone. On decomposition of the brucine salt with hydrochloric acid in the usual way a phthalate was obtained which had the following rotation in ether.

$$[\alpha]_D^{22} = \frac{+ 8.75^\circ \times 100}{1 \times 20} = + 43.75^\circ.$$

The acid ester ($[\alpha]_D^{22} = +43.75^\circ$) obtained as described above was dissolved in sodium hydroxide solution (3 mols) and the solution steam-distilled. The carbinol was extracted from the distillate with ether, the ether extract dried over anhydrous potassium carbonate, and the ether removed. The carbinol was then distilled at atmospheric pressure. It boiled at 138–140°, and analyzed as follows:

3.110 mg. substance: 8.180 mg. CO₂ and 3.395 mg. H₂O.

C₆H₁₂O. Calculated. C 72.00, H 12.00.

Found. " 71.72, " 12.21.

In ether it had the following rotation.

$$[\alpha]_D^{22} = \frac{+ 2.70^\circ \times 100}{1 \times 14.8} = + 18.2^\circ.$$

Reduction of Levo- Δ^5 -Hexenol to Levo-Methylbutyl Carbinol.

Levo-Methylbutyl Carbinol.—8 gm. of levo- Δ^5 -hexenol-(2) ($[\alpha]_D^{22} = -14.1^\circ$ in ether) obtained on decomposition of the mother liquors in the resolution of Δ^5 -hexenol-(2) were dissolved in 40 cc. of ether and reduced with hydrogen and colloidal palladium. Absorption of hydrogen was rapid and reduction was complete in 4 hours. The ether solution was dried over anhydrous potassium carbonate and the ether was removed. The carbinol was then distilled at atmospheric pressure. It boiled at $138-139^\circ$. It analyzed as follows:

5.185 mg. substance: 13.385 mg. CO_2 and 6.280 mg. H_2O .

$\text{C}_8\text{H}_{14}\text{O}$. Calculated. C 70.58, H 13.72.

Found. " 70.39, " 13.55.

In ether it had the following rotation.

$$[\alpha]_D^{22} = \frac{- 1.85^\circ \times 100}{1 \times 17.1} = - 10.8^\circ.$$

α -Naphthylurethane of Levo-Methylbutyl Carbinol (Hexyl- α -Naphthyl Carbamate).—The α -naphthylurethane of the carbinol obtained in the above experiment was prepared by heating 1 part of the carbinol with 1.7 parts of α -naphthyl isocyanate on the steam bath for 15 minutes. On standing overnight the urethane crystallized. After recrystallization from dilute alcohol, it melted at $78-81^\circ$ and analyzed as follows:

0.1000 mg. substance required 3.60 cc. 0.1 N HCl.

$\text{C}_{17}\text{H}_{21}\text{O}_2\text{N}$. Calculated. N 5.20. Found. N 5.04.

In absolute alcohol it had the following rotation.

$$[\alpha]_D^{22} = \frac{- 0.92^\circ \times 100}{2 \times 4.4} = - 10.4^\circ.$$

Conversion of Dextro- Δ^5 -Hexenol-(2) into Dextro-4-Hydroxyvaleric Acid.

Dextro-4-Hydroxyvaleric Acid.—32 gm. of dextro- Δ^5 -hexenol-(2) ($[\alpha]_D^{22} = +18.2^\circ$) were ozonized in 3 gm. lots in chloroform. A

solution of 3 gm. of carbinol in 40 cc. of chloroform was treated with ozone until a test portion no longer decolorized bromine in glacial acetic acid. The chloroform was removed under reduced pressure and the residue poured into water. After standing for 2 hours at room temperature with frequent shaking, the solution was heated under a reflux condenser on the water bath for 1 hour. After being cooled, alcohol was added until a clear solution was obtained.

In the subsequent treatment four lots of ozonized carbinol (3 gm. each) were combined and treated as follows: To the aqueous alcohol solution was added a solution of 90 gm. of silver nitrate in 200 cc. of water. The solution was stirred with a mechanical stirrer and a solution of 45 gm. of potassium hydroxide in 400 cc. of water was then slowly introduced. Stirring was continued for 5 hours after the addition of all the alkali. The solution was then filtered and carbon dioxide passed into the filtrate. The filtrate was concentrated on the water pump to a small volume, acidified to Congo red with 25 per cent sulfuric acid, and then extracted with ether in a continuous ether extractor. The ether extract was dried over anhydrous sodium sulfate and the ether removed. The residue was treated in ether solution with barium carbonate, filtered, and then neutralized rapidly with anhydrous potassium carbonate until no longer acid to litmus. The solution was filtered, the ether was distilled off, water was added, and the lactone converted into the barium salt. For analysis the barium salt was dried at 110° for 2 hours.

5.845 mg. substance: 3.695 mg. BaSO_4 .

$\text{C}_{10}\text{H}_{18}\text{O}_6\cdot\text{Ba}$. Calculated. Ba 37.2. Found. Ba 37.0.

In water the barium salt had the following rotation.

$$[\alpha]_D^{24} = \frac{+ 0.47^\circ \times 100}{2 \times 6.7} = + 3.5^\circ.$$

The rotation of the free acid was obtained by adding to 3.0 cc. of the above solution of barium salt 2.2 cc. of 0.5 N HCl. The rotation was observed immediately. For the free acid,

$$[\alpha]_D^{24} = \frac{+ 0.95^\circ \times 100}{2 \times 4.5} = + 10.5^\circ.$$

CRYSTALLINE UREASE.

III. VARIATIONS IN JACK BEAN MEAL AS AFFECTING THE YIELD OF CRYSTALS.

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It has been explained already that while certain samples of jack bean meal contain so much urease that extracts prepared according to our directions¹ furnish a good crop of urease crystals, other samples of jack bean meal are so poor in urease that no crystals can be obtained unless the dilute acetone extract is acidified with 10 cc. of 0.2 N acetic acid and allowed to stand in the ice chest overnight.²

We first stated that the 31.6 per cent acetone used to extract the 100 gm. of jack bean meal should have a temperature of 22°. Later, it was noticed that the crystals of urease that separated upon cooling were contaminated with less amorphous protein impurity if the temperature of the 31.6 per cent acetone was about 28°. We have tried extracting the meal with dilute acetone at 0°, keeping all of the apparatus in the ice chest. At this temperature the yield of urease crystals is somewhat increased, but the amount of amorphous protein³ impurity is greatly increased. If the acetone solution used to extract the meal is at 30°, the urease crystals are contaminated with needles of the globulin conca-*navalin* B. Hence, it was concluded that the best temperature for extraction is 28°. This applies to meal rich in urease.

Our custom has been to stir the jack bean meal with the dilute acetone for only 3 or 4 minutes. Experiments have shown that

¹ Sumner, J. B., *J. Biol. Chem.*, 1926, lxi, 435.

² Sumner, J. B., and Hand, D. B., *J. Biol. Chem.*, 1928, lxxvi, 149.

³ This material is a hitherto unreported jack bean protein. It is soluble in acid and in alkali, but is insoluble in neutral solvents.

if the material is stirred much longer the yield of urease crystals is diminished; if stirred for 1 hour the diminution is considerable. It seems probable that the urease exists in the jack bean combined with bases and that during the extraction certain acids in other cells of the bean are enabled to react with the bases combined with the urease. The higher the temperature, or the longer the stirring, the more opportunity there is for such combination to take place. A similar explanation is needed to show how it is possible to extract the globulin concanavalin A from jack bean meal with water. From aqueous extracts this globulin soon crystallizes and having done so can be shown to be extremely insoluble in the medium from which it has separated. It cannot be dissolved in any concentration of sodium chloride at room temperature, although it dissolves in saturated sodium chloride at 37°.

When the urease crystals are prepared from meal poor in urease, with acetic acid, as described in our directions, we have found that the mother liquor, after the urease crystals have stopped separating out, contains approximately 7 units of the enzyme per cc. Although the length of time that the material stands and the temperature of the ice chest have some effect, it can be said that the value of 7 units per cc. represents approximately the maximum solubility of urease under these experimental conditions. It is of very great interest to note that whenever the jack bean meal employed for the preparation of urease crystals is so poor in urease that the extract contains no more than 7 units per cc., no crystals can be obtained after addition of acetic acid and cooling. The same result can be achieved by inactivating or destroying a portion of the urease of the good meal by the addition of a sufficient amount of formaldehyde, mercuric acetate, or copper sulfate. Just as soon as the paralyser has been added in amount sufficient to destroy so much urease that the filtrate contains 7 units per cc., or less, it becomes impossible to obtain the urease crystals.

When the concentration of urease in the 31.6 per cent acetone filtrate is between 7 and 8 units per cc., a few urease crystals can be found by patient search with the microscope, and with still greater concentrations of urease in the filtrate the yield of crystals increases in proportion. We wish to point out that those who wish to prepare urease crystals cannot do so unless the dilute

acetone filtrate contains more than 7 units of enzyme per cc. It can be calculated from figures already given² that when the acetic acid is not added the filtrate must contain more than 12 units per cc. if crystals are to separate on cooling.

Within the past year we have purchased a variety of jack bean meal never before encountered in our work with urease. This meal contains urease that is only partly soluble in 31.6 per cent acetone and in 30 per cent alcohol. Thus, although there is sufficient urease in the meal (100 units per gm.) to give a fair crop of crystals, no crystals can be obtained and the filtrates have been found to contain between 6 and 7 units of urease per cc. Although dilute acetone and dilute alcohol do not extract the urease in a satisfactory manner, distilled water has been found to extract it nearly as well as it extracts the urease from samples of the

TABLE I.

Meal No.	Urease units per:			
	Gm. meal.	Cc. water extract.	Cc. acetone extract.	Cc. alcohol extract.
280328	100	20.0	6.6	7.4
F 48	100	20.2	6.9	7.4
F 18	102	21.5	10.3	12.2

ordinary meal. Inasmuch as we have been sold meal of this unusual nature from two different supply houses, it is likely that the fault lies with the jack beans that have been sold recently for the manufacture of meal. Table I gives figures showing the units of urease per gm. of one sample of satisfactory meal and two samples of unsatisfactory meal. The table shows how many units of urease are capable of being extracted by distilled water, by 31.6 per cent acetone, and by 30 per cent alcohol. In each case 1 part of the meal by weight was mixed with 5 volumes of the solvent. The water extract was centrifuged, the acetone extract was filtered in the ice chest, while the alcohol extract was filtered at room temperature because chilling precipitates urease from 30 per cent alcoholic extracts.

We have attempted to improve the extraction of urease from the unsatisfactory meal by adding to the 31.6 per cent acetone small quantities of calcium chloride, calcium acetate, magnesium

sulfate, potassium oxalate, and saponin, but without success. The calcium and magnesium salts almost entirely prevented the extraction of the urease; the potassium oxalate and the saponin were without effect.

A curious property shown by the unsatisfactory meal is decreased hydration capacity. When one treats 100 gm. of normal jack bean meal with 500 cc. of 31.6 per cent acetone, pours on a filter, covers with a filter paper to prevent undue evaporation, and allows the material to filter overnight in an ice chest, the volume of the filtrate is always between 310 and 315 cc. However, with the unsatisfactory meal the volume of the filtrate is from 325 to 330 cc. This decreased hydration capacity may be concerned with the decreased solubility of the urease.

THE INORGANIC PHOSPHATE CONTENT OF RESTING MAMMALIAN MUSCLE.

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(Received for publication, July 5, 1928.)

In 1927 Fiske and Subbarow (5) found in mammalian muscle a compound of phosphoric acid which underwent rapid hydrolysis in acid solution. Almost simultaneously Eggleton and Eggleton (2) reported a similar compound in frog muscle and recently (3) they have confirmed the findings of Fiske and Subbarow on mammalian muscle. From their data Fiske and Subbarow conclude that the inorganic phosphate content of striated muscle of the cat is 20 to 25 mg. per cent (expressed as P) instead of the usually given values of 80 to 100 mg. per cent. Eggleton and Eggleton report 26 mg. per cent for the gastrocnemius muscle of the rabbit and 58 for this muscle in the guinea pig.

The method used by both groups of workers was the comparison of the rate of color development of a trichloroacetic acid muscle filtrate with that of a standard phosphate solution when these were treated simultaneously with molybdic acid and a reducing agent, and to extrapolate the values to zero time. Neither group of investigators has stated specifically the method of obtaining the muscles from mammals; that is, whether the animal was killed and the muscle excised, or whether the muscle was removed from the anesthetized animal.

This method of determining the phosphate content of muscle is subject to several criticisms. In the first place, the extrapolation to zero time assumes that the reactions involved are all of the first order. Second, in attempts to repeat the work of Fiske and Subbarow it was found that during the first 10 minutes, when the greatest change is taking place, the color developed in the solution containing muscle filtrate was olive to blue-green instead

of the pure blue of the standard. Furthermore, the values obtained were found to fall on an approximately logarithmic curve, as is shown in the charts of Irving and Wells (7), instead of being nearly on a straight line, as is claimed by Fiske and Subbarow. Finally, one cannot be certain that muscle removed post mortem or muscle excised from the living animal and then frozen has not undergone appreciable change from the conditions which existed *in vivo*.

In order definitely to establish the normal range of the inorganic phosphate of resting mammalian muscle and to provide a technique suitable for the study of the labile compound, a series of determinations was made on the resting gastrocnemius muscle of the rabbit, guinea pig, rat, cat, and dog by precipitating the inorganic phosphate from protein-free muscle filtrates with magnesia mixture and determining phosphorus in the precipitate. Previous workers (5, 8) have shown that the labile compound does not undergo hydrolysis in alkaline solution in the absence of muscle enzymes, and it is not precipitated by magnesia mixture.

The samples were obtained by freezing the muscle with carbon dioxide snow and ethyl chloride while the animal was under amytal anesthesia, as previously described by Davenport and Davenport (1). The muscle was carefully dissected away from surrounding structures, with the circulation and nerves intact. The skin incision was then closed with wound clips and the animal allowed to rest for 5 or 10 minutes. The clips were then removed, the tendon of insertion cut, and carbon dioxide snow packed around the distal portion of the muscle. Ethyl chloride was then dropped on the snow until a slush formed, and this slush was worked toward the origin as the distal parts became frozen. In this way the circulating blood was frozen in with the muscle and there was no temporary asphyxia of the unfrozen parts. The muscle was not removed from the animal until it was completely frozen. In the majority of cases, except with rats, there was no evidence of contracture and usually only very slight twitching. The rat muscles, however, did twitch considerably. With excised muscles there was generally a marked contracture. Gorodissky (6) reports that when frog muscles are frozen in liquid air there is always a contracture. With regard to the apparently higher irritability of rat muscles, it may be noted that the lactic acid content of the

resting muscle in this species is found to be consistently higher than in the other animals investigated (Davenport and Davenport (1) and unpublished data of G. L. Foster).

Some preliminary experiments in which saturated ammonium sulfate solution was used as the protein precipitant gave values of 20 to 25 mg. per cent for the gastrocnemius muscle of the rabbit. This precipitant was abandoned, however, on account of the difficulty of making lactic acid determinations on the filtrates, and the following procedure was adopted.

The frozen muscle was cut into slices from 0.1 to 0.2 mm. thick. These were immediately put into a small beaker which was kept cold by surrounding it with carbon dioxide snow. When the entire muscle had been sliced, all the material was put into a weighed flask containing 5 per cent trichloroacetic acid solution cooled to 0°. The whole was shaken well, the flask and contents reweighed, and after 10 minutes of constant shaking the liquid was poured into a cold filter. It was found, in agreement with Eggleton and Eggleton (2), that equilibrium to the extent of at least 98 per cent was reached under these conditions.

Immediately after filtration was complete, a measured volume of the filtrate—usually 10 cc., equivalent to 0.7 to 1.0 gm. of muscle—was pipetted into a tapered centrifuge tube containing 0.5 cc. of concentrated ammonia solution. 2 or 3 cc. of magnesia mixture were then added and the sides of the tube rubbed down with a rubber policeman. After standing an hour to insure complete precipitation, the tube was again rubbed down, centrifugalized, the liquid decanted, and the tube inverted on filter paper to drain. The precipitate of magnesium ammonium phosphate was dissolved by the addition of 2 drops of 5 N sulfuric acid and phosphorus determined by the method of Fiske and Subbarow (4). It is necessary to proceed as rapidly as possible in the preparation of the sample because appreciable hydrolysis of the labile compound takes place in trichloroacetic acid solution even at 0°, as shown previously by others (2, 5).

By the procedure described above it was found that the inorganic phosphate content of resting skeletal muscle of the cat, rat, and guinea pig is slightly over 20 mg. per cent and that of the dog and rabbit is somewhat under this figure.

Lactic acid determinations were made on the filtrates wherever

TABLE I.
*Inorganic Phosphate and Lactic Acid in Muscles of Rabbits, Guinea Pigs,
Rats, Cats, and Dogs.*

Animal No.	Sample No.	Kind of muscle.*	Inorganic P. <i>mg. per 100 gm.</i>	Lactic acid. <i>mg. per 100 gm.</i>	Remarks.
Rabbit.					
1	1	G.r.	19	15	Spinal block with alcohol after amytal analgesia. Same as Sample 1 except allowed to stay 30 min. at 0° before neutralization.
	2	" l.	16	19	
	1 a	" r.	21		
2	3	G.r.	18	8	Respiration nearly normal.
	4	" l.	15	7	
3	5	G.r.	19	24	G.r. frozen during slowed respiration; G.l. frozen just as respiration stopped. 3 min. post mortem. Muscle twitched violently during freezing.
	6	" l.	23	27	
	7	T.l.	53	155	
4	8	G.r.	19	26	Died at beginning of freezing G.r. G.l. frozen 7 min. post mortem.
	9	" l.	47	148	
5	10	G.l.	49	86	10 min. post mortem.
	11	" r.	78	108	15 " " "
	12	T.l.	89	163	1½ hrs. " " "
	13	" r.	91	180	1½ " " " "
6	14	G.r.	100	230	1½ hrs. post mortem. P determined on magnesia mixture precipitates.
	15	" l.	101	250	
6	14	G.r.	102		Direct colorimetric determination on trichloroacetic acid filtrates.
	15	" l.	104		
Guinea pig.					
11	21	G.l.	26		Considerable hemorrhage after freezing G.l.
	22	" r.	29		
12	23	G.r.	32		Respiration failed while freezing G.r.; G.l. frozen immediately post mortem.
	24	" l.	40		

* G. = gastrocnemius; S. = soleus; T. = triceps brachii; r. and l. = right and left.

TABLE I—*Concluded.*

Animal No.	Sample No.	Kind of muscle.*	Inorganic P.	Lactic acid.	Remarks.
			mg. per 100 gm.	mg. per 100 gm.	
13	25	r.	20	7	Mixed muscles of hind leg.
	26	l.	Lost.	8	
14	27	r.	22	18	Mixed muscles of hind leg.
	28	l.	21	15	
Rat.					
21	31	G.l.	22		Considerable twitching.
	32	" r.	24		" "
22	33	G.r.	22		" "
	34	" l.	26		Some thawing of slices.
23	35	r.	22	15	Mixed muscles of hind leg.
	36	l.	22	19	
Cat.					
31	41	G.r.	21	13	
	42	" l.	21	13	
32	43	G.l.	22	17	
	44	" r.	22	17	
Dog.					
41	51	GS.l.	16	14	
	52	" r.	17	14	
42	53	GS.r.	18	9	
	54	" l.	17	12	

sufficient material was available. Phosphorus determinations on muscles frozen at various times after the death of the animal were made also. Included in Table I is a comparison of the values obtained by direct colorimetric determinations made on muscles removed post mortem with those obtained on magnesia mixture precipitates from the same filtrates.

It will be seen that there is a rapid increase in the inorganic phosphorus values very quickly after death as well as a marked increase in the lactic acid content. No definite ratio between the

increases in phosphorus and lactic acid can be deduced from the data available.

The values for inorganic phosphorus reported here are probably higher than the actual ones, as some hydrolysis takes place while the muscle is in contact with the trichloroacetic acid and during the filtration. The attempt has been made to keep this hydrolysis at a minimum by keeping the solution at 0° or lower and working as rapidly as conditions will permit until the filtrate is neutralized. Constant and vigorous shaking during the 10 minutes allowed for extraction is necessary in order to obtain equilibrium between the muscle and the solution. Experiments were performed in which portions of the solution were withdrawn for analysis after 5, 10, and 30 minutes contact between the muscle and the trichloroacetic acid solutions. It was found that the 5 minute samples gave values both for phosphorus and lactic acid which were about 95 per cent of those obtained at 10 and 30 minutes.

SUMMARY.

1. The inorganic phosphate content of resting muscle is slightly over 20 mg. per cent for the cat, rat, and guinea pig and slightly under this figure for the rabbit and dog.

2. The amount of inorganic phosphate increases very rapidly after death just as does the lactic acid.

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CHEMICAL STUDIES OF MUSCLE CONTRACTURE.

I. THE LACTIC ACID CONTENT.

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An exact knowledge of the relationship of lactic acid to muscle shortening would contribute much toward the subject of muscle physiology. Data to show that lactic acid is an important intermediary product of the carbohydrate metabolism of muscle are available in abundance, but the time relationship between lactic acid formation and the act of shortening is not yet clear.

Recent observations on rigor mortis (8, 9, 21), as well as the classical observations on crustacean muscle by Claude Bernard, have shown that rigor can occur with so little acid present that the muscles may be neutral or even slightly alkaline.

Attempts have been made by Bethe and coworkers (1) to show that in order to include all the known facts in one hypothesis, which would be generally applicable to muscle shortening, it is necessary to assume the existence of a non-acid "Verkürzungssubstanz." The findings of Meyerhof (13) and of Matsuoka (11) have led them to the conclusion that the development of tension in muscle is always associated with the production of lactic acid. Riesser and Heianzan (16) have pointed out that among the contractures produced by drugs or chemical agents the contracture caused by acetyl choline acting on isolated frog muscle was an exception and not accompanied by a measurable increase of lactic acid. The opposite finding, in principle, has been reported by Gasser and Dale (6). They found a measurable increase in the lactic acid content in the denervated gastrocnemii of cats when these muscles developed tension from acetyl choline excitation.

Zondek and Matakas (22) found no increase in the lactic acid production of isolated gastrocnemii of frogs during drug contractures of short duration, and concluded that the increased production of lactic acid during drug contractures observed by others was due to injury to the muscle. They have pointed out that the production of sustained shortening *in vitro*

without increase of lactic acid is proof that lactic acid does not cause the shortening. Embden and coworkers (4) have obtained data which indicated that a muscle excited by a single stimulus becomes more alkaline at the moment of shortening, and that the formation of lactic acid occurs during the phase of relaxation.

Experiments made by Swartz and Oschmann (20) with the action of monobromoacetic acid on isolated frog gastrocnemii showed no increase in the lactic acid content of the muscles during the contracture produced by this drug.

In the work to be described, we wished to ascertain how the lactic acid content of muscles in a state of contracture caused by tetanus toxin would compare with that of muscles obtained from normal animals. The production of localized tetanus can be accomplished easily in experimental animals by intramuscular or subcutaneous injection of the toxin of *Bacillus tetani*. The muscles nearest the site of injection become hypertonic in 3 to 7 days and the development of tension is sufficient to immobilize the affected muscles or, in the case of a limb, to immobilize the entire extremity. The course of the development of this type of contracture has been studied by Meyer and Ransom (12) and later by Ransom and Morris (14). They have found that the muscle shortening is at first dependent upon intact innervation but later persists under deep anesthesia or even after section of the motor nerve. The first type of contracture has been designated as "hypertonic" and may be considered reversible in character because relaxation occurs during anesthesia or after section of the motor nerve. The final type which represents a permanent shortening of resting muscle has been termed "myostatic" contracture (15). It must be due to some permanent change in the muscle substance because it persists after section of the motor nerve.

The cause of this irreversible contracture might conceivably be found in the chemical constituents of the muscle and the most obvious of these was considered to be lactic acid.

EXPERIMENTAL.

Guinea pigs weighing about 550 gm. were injected aseptically in the popliteal space of the right leg with approximately one-half of one M.L.D. of tetanus toxin.¹ Contracture which persisted

¹ We are indebted to Parke, Davis and Company for a supply of tetanus toxin.

under deep anesthesia developed in 5 to 7 days. When the shortening had become irreversible the gastrocnemii were removed for analysis. The muscle on the uninjected side served in a way as a control, although it was usually slightly involved.

In the determination of lactic acid in mammalian muscle the method of preparing the samples for analysis becomes one of prime importance. The technique for obtaining such samples has been reported (2). It consists essentially of freezing the muscles with the blood circulation intact by a mixture of CO₂ snow and ethyl chloride,² removal from the animal, and then cross sectioning them into slices about 0.1 mm. thick. The slices are transferred while still frozen into ice-cold 4 to 5 per cent trichloroacetic acid. Weights are obtained by weighing the vessel (a 100 or 150 cc. extraction flask) before and after it receives the muscle sample.

Table I shows the amounts of lactic acid found with varying experimental procedures. The determinations were made by the Friedemann, Cotonio, and Shaffer procedure (5) with colloidal manganese dioxide for the oxidizing agent, and apparatus modified to determine quantities between 0.05 and 2.0 mg. (2, 3).

Since the animals must be anesthetized, for humane reasons and to prevent rise of lactic acid, from struggling, the effect of the anesthetic has to be considered. Ether seemed obviously unsuited because it causes an acidosis, and this acidosis is associated with an increase of lactate ion in the blood (17). Urethane was tried in two animals, but caused cyanosis and gave higher lactic acid values for muscle than did amytal. The latter gave values more nearly like those found for blood in resting mammals.

Since the lactate ion is diffusible, one would expect an equilibrium between muscles and blood while an animal was in a state of rest. The blood lactic acid has been found to be between 10 and 20 mg. per cent (7, 18) in resting mammals, and it seems significant that this corresponds with the range of the lactic acid con-

² It has been found most convenient to pack the snow about the distal portion of the muscle and then by "wetting" the snow with ethyl chloride to cause the freezing mixture so formed to flow first around the portion adjacent to the insertion. By elevating the foot and pushing the freezing mixture toward the origin, the muscle can be frozen solid with a minimum amount of stimulation and without premature arrest of the circulation.

tent of gastrocnemii of guinea pigs and rats (14) (as well as unreported data on rabbits and cats) by freezing the muscles *in situ* while the animals were anesthetized with amytal.

It seems reasonable to conclude that the general anesthetic which gives the lowest values is the one which maintains a physiological condition most nearly true to normal rest, because the known disturbances to muscle metabolism cause a rise in lactic acid. There is still the possibility, however, that amytal exerts a specific depressing action upon the formation of lactic acid. We have attempted to throw some light upon this phase of the question by removing muscles during regional anesthesia produced by blocking the spinal cord in the sacral region. It is fairly easy to enter the spinal canal of a guinea pig with a small needle and inject about 0.05 cc. of absolute alcohol with a tuberculin syringe. If local anesthesia is induced first with about 0.1 cc. of 1 per cent novocaine injected into the skin and deeper tissues, the final injection of alcohol can be accomplished without serious disturbance to the animal. Values obtained by this method of anesthesia are somewhat higher than those obtained with amytal. This does not necessarily prove that amytal depresses the formation of lactic acid other than by inducing muscular rest, because the anterior portion of the animal was active. This activity may have given rise to sufficient lactate to raise the content in the posterior portion of the body by diffusion from the blood stream. A point against the possibility that amytal may depress the formation of lactic acid has been furnished by Long (10) in finding that muscle glycogen decreases during 3 hours of general anesthesia. If this decrease is accompanied by a fall in body temperature and no increase in lactic acid nor blood sugar, then the fate of the glycogen becomes a problem in itself worth investigating.

The findings appear to be unquestionably negative relative to the possibility of there being a storage of lactate ion in the muscle during irreversible contracture caused by tetanus toxin, or that lactic acid is being formed in order to maintain the shortening.

Muscles removed from animals anesthetized with amytal ranged from ± 8 to 24 mg. per cent on the control side and from ± 8 to 30 mg. on the side of the contracture. There was no consistent difference between the two sides, and the values obtained for

TABLE I.

Lactic Acid Found in the Gastrocnemii of Guinea Pigs and Rabbits during Contracture Caused by Tetanus Toxin.

Animal No.	Con- dition of mus- cle.*	Lactic acid. <i>mg. per 100 gm.</i>	Anesthetic.	Sciatic nerves.	Remarks.
Guinea pig.					
2	F.	18	Amytal.	Intact.	
	C.	12			
3	F.	15			
	C.	23			
4	F.	15		Cut.	{ Lactic acid present in quan- tity too small to determine with accuracy.
	C.	15			
5	F.	13			
	C.	± 8			
7	F.	10			
	C.	17			
8	F.	<8			
	C.	<8			
10	F.	<8			
	C.	<8			
11	F.	14			
	C.	15			
12	F.	28	Urethane.	Intact.	Muscles cyanotic.
	C.	26			
13	F.	39			
	C.	35			
15†	F.	16	Spinal block.	Intact.	{ Animal struggled violently with anterior portion of body.
	"	21			
16	"	32			
	C.	40			
17	F.	21		Cut.	
	C.	19			
18	F.	18			
	C.	18			
Rabbit.					
21	F.	24	Amytal.	Intact.	Animal shivered for about $\frac{1}{2}$ hr.
	C.	30			
22	F.	23			
	C.	16			
23	F.	12			
	C.	15			

* F. = flaccidity; C. = contracture.

† Normal animal.

both sides correspond closely with those obtained in normal animals under comparable conditions (2). There is a considerable range in values from animal to animal (greater than the range from side to side in the same animal). This variation is due probably to variations in respiration and circulation in animals under general anesthesia, and to the struggling in the anterior half of the body during spinal block.

Since lactic acid forms rapidly in a muscle which has had its circulation arrested, previous values for resting mammalian muscle have been too high. Values closely approaching the resting content have been reported by Gasser and Dale (6) and by Simpson and MacLeod (19). The former found 35 mg. per cent, and the latter 24 mg. in muscles removed from living cats.

It is possible that the genesis of the contracture is related to an excessive formation of lactic acid by the muscle, and that later, when the contracture becomes independent of the nerve supply to the muscle, a condition of lactate content indistinguishable from resting values returns. The methods used were such that the latter possibility was neither eliminated nor strengthened.

In some animals the sciatic nerve was cut 20 to 30 minutes before the muscles were frozen. In others the muscles were frozen with the nerve supply intact. A survey of Table I will show that the quantities of lactic acid found were lower and more uniform when the nerve had been sectioned. The somewhat higher and more irregular values obtained when the nerve was intact seem attributable to reflex twitching of the muscle due to excitation of sensory nerves by the freezing.

In one observation not included in Table I, the sciatic nerves were stimulated by faradization for a period of 2 minutes at intervals of 10 seconds "on," alternating with 5 seconds "off." The flaccid muscle was tetanized first and frozen within 20 seconds after the stimulation was stopped. Freezing was begun just as the stimulation was stopped. After removing the "normal" muscle, the tonic muscle was stimulated and frozen in a similar manner. The lactic acid found was: flaccid muscle, 92 mg. per cent; tonic, 70 mg. per cent. This observation is cited to show (in at least a qualitative manner) that the muscle in contracture is capable of forming lactic acid when its motor nerve is stimulated. It is capable also of forming lactic acid post mortem, and in these two phenomena does not differ qualitatively from the normal muscle.

SUMMARY.

The lactic acid content of muscles which have undergone permanent shortening from treatment with tetanus toxin lies within the same range as that of normal muscles either from the flaccid side of the same animal or from normal animals.

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SOME BIOCHEMICAL RELATIONS OF PHENOLS.

II. THE EFFECT OF HYDROQUINONE ON THE VITAMIN A CONTENT OF STORED OILS.

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INTRODUCTION.

In the first article of this series (1), it was shown that hydroquinone has a definite beneficial effect when used as a constituent of certain diets. At that time it was stated that a series of studies was in progress dealing with the protective action of phenols toward vitamins. This is the first of a series of articles describing these studies.

Hopkins in 1920 (2) stated that the antixerophthalmic factor is destroyed by aeration for 4 hours at 120°. He also found that at 15-25° the destruction proceeded when the oil was exposed to the atmosphere in thin layers. Drummond and Coward (3) in the same year stated that the destruction of the vitamin under similar conditions of temperature is probably due to oxidation. In 1914, Kraus (4) reported that the oxidation in cod liver oil emulsions is induced by emulsifying agents. Drummond, Silva, and Coward (5) later found that cod liver oil emulsified with Irish moss, or gum acacia, which contain oxidases, gradually loses its vitamin A potency.

That this destructive oxidation is accompanied by a simultaneous oxidation of the fatty acids is indicated by the work of Powick (6) who found that mixing the vitamin with a rancid fat accelerated the rate of destruction. He attributed this increased rate to the oxidation of the vitamin by the organic peroxides of the rancid fat.

In view of this suggested relationship between the rate of destruction and peroxide formation, it might be expected that the "antioxysens" of Moureu and Dufraisse (7) would retard the destruction of the vitamin. Lund (8) found that the rancidity of cod liver oil was delayed in the presence of easily oxidizable substances such as liver debris, or pyrogallol. Mattill (9) studied the relation of the acetyl number of fats and the protective relation of the hydroxyl group. He emphasizes the importance of the recognition of the rôle played by oxidizing catalysts and antioxidants in the biological assay of foods for the vitamin content.

508 Biochemical Relations of Phenols. II

TABLE I.
*Animals Receiving Milk Fat with Hydroquinone.**

Body weights are given in gm.

Wks. on diet.	Rat 37.	Rat 42.	Rat 53.	Rat 54.	Rat 55.
	64.5	43.5	50.0	52.5	40.5
1	83.2	54.0	63.2	66.8	57.6
2	107.1	66.0	79.5	82.0	79.0
3	118.7	82.4	91.9	90.6	92.8
4	135.0	96.0	107.2	99.0	95.9
5	148.4	105.6†	108.5†	96.4†	93.7†
6	152.8	118.8	136.0	113.4	106.8
7	150.7	146.8	161.5	130.0	130.9
8	144.8†				
9	155.3				
10	192.7				

Animals Receiving Milk Fat without Hydroquinone.†

Wks. on diet.	Rat 43.	Rat 44.	Rat 45.	Rat 47.	Rat 48.	Rat 49.	Rat 56.
	40.0	40.5	54.5	60.0	49.0	50.0	41.0
1	56.3	55.7	63.5	77.7	62.0	66.8	60.4
2	76.7	77.0	78.1	98.3	78.0	83.5	81.8
3	92.4	91.7	97.7	115.1	90.7	95.0	96.2
4	110.0	104.5	99.0†	130.0	98.9	103.3	108.7
5	114.1†	103.9†	104.0	136.0	94.0†	110.4	113.7
6	93.6	94.0	89.0	145.5†	99.2	110.0†	112.0†
7	85.5	§	§	151.8	§	112.4	115.5
8	§			151.0		107.2	95.7
9						92.7	§
						§	

* All of these rats showed ophthalmia the week preceding the change in diet. All were discarded when recovery was complete.

† Indicates time of transfer.

‡ All of these rats except Rats 45 and 47 showed ophthalmia the week preceding the change of diet. Rat 47 was discarded because of lack of food; slight ophthalmia at time of discarding.

§ Indicates death.

EXPERIMENTAL.

Feeding Experiments.

Experiment 1.—Young albino rats at 28 days of age were placed upon the McCollum Diet 3626 (10) modified in that the milk fat

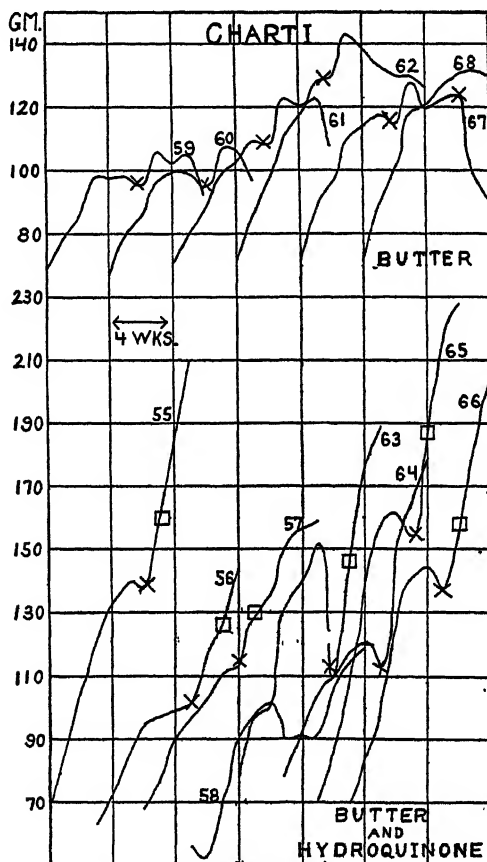


CHART I. Animals 57 and 58 developed a lung infection in the 6th week and failed to show the usual growth for a time. X ophthalmia when removed from preliminary diet. □ recovery from ophthalmia.

was replaced by an equal amount of hydrogenated cottonseed oil. McCollum's Salt Mixture 20 was used. These rats were main-

tained upon this diet until there was unmistakable evidence of ophthalmia and a definite break in the growth curve. At this time (5 to 7 weeks) the animals were transferred from this ophthalmia-developing ration to diets in which one-half of the hydrogenated cottonseed oil was replaced by milk fat stored for 46 days. One group of animals received milk fat stored at 37° in an open beaker out of direct sunlight. Another group received milk₁ fat

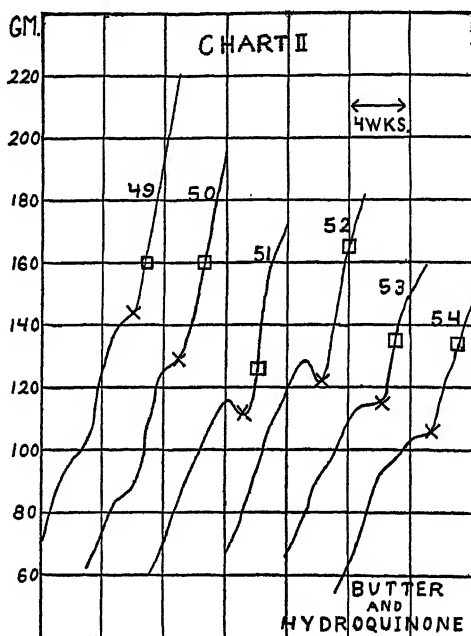


CHART II. X ophthalmia when removed from preliminary diet. □ recovery from ophthalmia.

stored under identical conditions with the exception that it contained 0.5 mg. of hydroquinone per cc. of fat. The animals were maintained on these diets until death ensued or until the growth curve and appearance of the eyes indicated complete recovery. Tabular results of this experiment are presented in Table I.

Experiment 2.—In this experiment animals also received McCollum's Diet 3626 modified as in Experiment 1, and as in the preceding experiment were transferred to modified rations containing

milk fat stored with and without hydroquinone. The preliminary period of feeding in this experiment was uniformly 6 weeks. In this case, however, the period of storage was 37 days and the hydroquinone content of the preserved fat was 1.0 mg. per gm. The results are presented in Chart I.

Experiment 3.—Rats were kept on the preliminary diet 6 weeks and then placed on a diet in which one-half of the hydrogenated

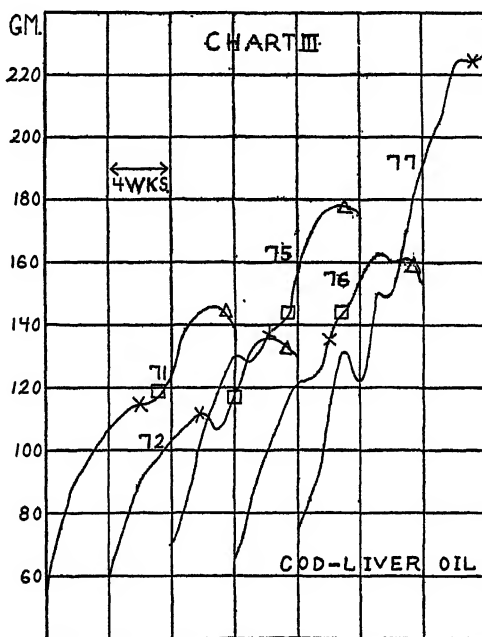


CHART III. × ophthalmia when removed from preliminary diet. □ recovery from ophthalmia. Δ reappearance of ophthalmia.

cottonseed oil was replaced with the same lot of milk fat as was used in Experiment 2, which had been stored at 37° for a period of 83 days, with hydroquinone in the ratio of 1 mg. per gm. All of the animals had developed ophthalmia at the close of the preliminary period. The growth curves are shown in Chart II.

Experiment 4.—As in the preceding experiments, animals were fed the preliminary ophthalmia-producing diet for 6 weeks, after

which time they were transferred to diets in which one-fifth of the hydrogenated cottonseed oil was replaced by stored cod liver oil. These samples of cod liver oil were stored under the same conditions as described in the case of milk fat in Experiment 1, for a period of 56 days. The hydroquinone content of the treated oil was 1.0 mg. per gm.

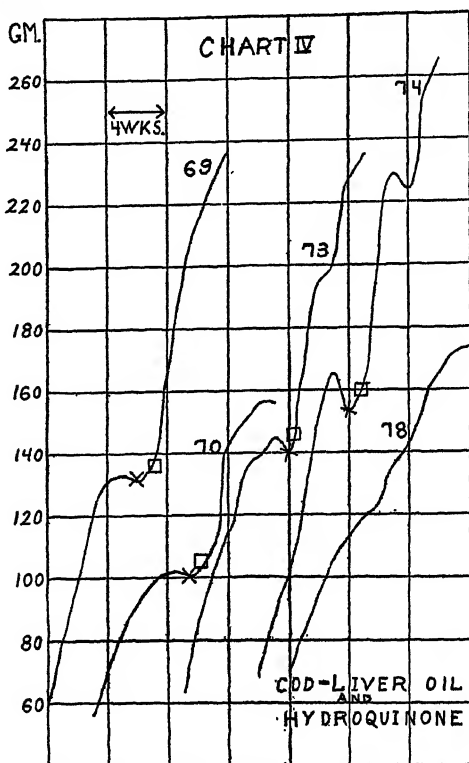


CHART IV. X ophthalmia when removed from preliminary diet. □ recovery from ophthalmia.

All animals receiving cod liver oil plus hydroquinone were cured of ophthalmia inside of 3 days. Those which received cod liver oil without hydroquinone recovered more slowly but all were cured by the close of the 10th day.

At the end of 4 weeks the animals were placed on diets in which

only one-tenth of the hydrogenated cottonseed oil had been replaced by cod liver oil which had been stored 132 days. All those receiving untreated oil developed ophthalmia within 2 weeks, while those receiving preserved oil were entirely free from eye soreness.

The growth curves are shown in Charts III and IV.

Color Tests for Vitamin A.

Portions of cod liver oil and of milk fat with and without hydroquinone were placed in Erlenmeyer flasks and stored in contact with air, but out of direct sunlight, at a temperature of 37°. The

TABLE II.
Color Tests for Vitamin A.

Mg. hydroquinone per 50 cc. fat.	SbCl ₃ test on cod liver oil after 40 days.*	AsCl ₃ test on cod liver oil after 40 days.*	SbCl ₃ test on milk fat after 83 days.†	AsCl ₃ test on milk fat after 83 days.†
0.0	Red-brown.	Brown.	Pale brown.	Pale brown.
1.0	" "	"	Very faint blue.	" "
5.0	Pale brown.	"	" " "	Very faint blue.
10.0	Faint blue.	Very faint blue.	" " "	" " "
25.0	Good "	Faint blue.	" " "	Faint blue.

The presence of hydroquinone in the fresh cod liver oil and in the fresh milk fat had no effect on these color tests.

* The fresh cod liver oil gave a strong blue color.

† The fresh milk fat gave a faint blue color.

flasks were shaken daily. The SbCl₃ color test of Carr and Price (11) and the AsCl₃ color test of Rosenheim and Drummond (12) were made on the fresh fats. The tests were repeated at the end of 40 days in the case of cod liver oil and at the end of 83 days in the case of milk fat. The results of these tests are shown in Table II.

Chemical Constants.

Three lots of milk fat, Lot A without hydroquinone, Lot B containing 0.1 mg. of hydroquinone per gm. of fat, and Lot C containing 0.5 mg. of hydroquinone per gm. of fat, were placed in

TABLE III.
Chemical Contents of Milk Fat.

Lot.	Fresh fat.	After 30 days.	After 60 days.	Change in 60 days.
Iodine No. (Hanus).				
A	32.29			
	32.21	31.80	27.72	
	32.14	31.81	27.52	
Average.	32.21	31.80	27.62	-4.59
B	32.17	32.52	32.39	
	32.01	32.36	32.26	
Average.	32.09	32.44	32.33	+0.24
C	32.24			
	31.91	32.38	31.95	
	32.05	32.59	32.11	
Average.	32.07	32.47	32.03	-0.04
Saponification No.				
A	230.11	231.66	237.25	
	229.82	232.14	237.53	
Average.	229.97	231.90	237.39	+7.42
B		231.05		
	230.04	231.06	232.00	
	230.17	230.94	232.03	
Average.	230.11	231.02	232.01	+1.90
C			231.89	
	230.62	232.60	232.21	
	230.98	231.98	232.34	
Average.	230.80	232.29	232.14	+1.34

TABLE III—*Continued.*

Lot.	Fresh fat.	After 30 days.	After 60 days.	Change in 60 days.
Reichert-Meissl No.				
A	29.37	30.50	30.44	+0.66
	29.86	30.47	30.18	
			30.22	
Average.	29.62	30.48	30.28	
B	29.85	29.35	29.41	-0.30
	29.78	29.55	29.64	
Average.	29.82	29.45	29.52	
C	29.98	30.30	29.87	-0.18
	30.38	30.46	30.13	
Average.	30.18	30.38	30.00	
Acid No. (mg. KOH per gm.).				
A	0.727	0.807	2.122	+1.390
	0.709	0.793	2.094	
Average.	0.718	0.800	2.108	
B	0.752	0.782	0.764	+0.004
	0.757	0.767	0.753	
Average.	0.755	0.774	0.759	
C	0.793	0.806	0.839	+0.028
	0.825	0.823	0.836	
Average.	0.809	0.814	0.837	

TABLE III—*Concluded.*

Lot.	Fresh fat.	After 30 days.	After 60 days.	Change in 60 days.
Acetyl No.				
A	4.50	6.01	9.91	
	4.54	5.77	9.99	
Average.	4.52	5.89	9.95	+5.43
B	6.01	5.65	4.03	
	5.98	6.14	3.94	
Average.	6.00	5.90	3.99	-2.01
C	5.04	4.94	3.00	
	4.94	4.94	2.63	
Average.	4.99	4.94	2.82	-2.17

We are unable to explain the consistent drop in the acetyl numbers in the case of both Lots B and C.

The color of the milk fat in Lot A gradually faded during storage while that of Lots B and C remained unimpaired.

Lot C at the end of 60 days darkened during the treatment involved in the determination of the Reichert-Meissl number, the saponification number, the acid number, and the acetyl number.

Erlenmeyer flasks and stored in contact with air but out of direct sunlight at a temperature of 37°. The flasks were shaken daily. Portions of each lot were analyzed at the beginning of the period of storage, at the expiration of 30 days, and at the end of 60 days. The iodine number, saponification number, Reichert-Meissl number (13), acid number (14), and acetyl number (15) were determined. The results are shown in Table III.

DISCUSSION.

Feeding Experiments.

The experiments show a definite protective action against destruction of vitamin A by hydroquinone in both milk fat and cod liver oil, when these are stored a number of weeks under the stated conditions. Drummond and Coward (3) found that butter lost

power to restore growth when heated at 37° for 3 weeks. It is worthy of note that the fourteen animals fed milk fat containing hydroquinone resumed growth and recovered from ophthalmia without exception. We suggest as does Mattill (9) that Quinn's (16) assumption that vitamin A may exist in more than one form in nature, may be based on observations affected in part by the presence of certain natural occurring antioxygens.

Color Tests and Chemical Constants.

Application of color tests to the stored fats substantiates the results obtained in the feeding experiments. The chromogenic substance of milk fat appears to be more resistant to oxidative destruction, in the presence of hydroquinone, than does that of cod liver oil. Possibly this is due to the greater unsaturation and resulting readiness of peroxide formation of the latter. It is interesting also to note that hydroquinone serves to preserve the natural pigments of the milk fat.

The determined chemical constants likewise confirm the preservative action of hydroquinone, as would be expected from the results of the work of Moureu and Dufraisse (7). There is indicated an optimum concentration of hydroquinone, the exact value of which, for butter fat and other oils, is the subject of further study.

This laboratory has under way a series of investigations of the preservative action of hydroquinone on vitamins A and D in natural oils and in these oils when in the emulsified state and in food mixes.

These studies were made possible by a grant from the Agricultural Experiment Station of Michigan State College.

CONCLUSION.

This series of experiments shows a definite antioxygenic effect of hydroquinone on vitamin A of milk fat and of cod liver oil. There is also shown a correlation between the preservative action of hydroquinone on vitamin A and the protection of milk fat from decomposition.

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PERIPLOCYMARIN AND PERIPLOGENIN.

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Closely allied to the family of Apocynaceæ, which embraces a number of plants yielding heart poisons such as the strophanthins, is the family of Asclepiadaceæ. Belonging to the latter is a species, *Periploca græca*, found principally in the region of the Black Sea and some of the eastern Mediterranean countries, which has been shown by Lehmann¹ to contain a glucoside, *periplocin*. This substance was isolated by this worker in pure form as needles which were soluble in alcohol and water and practically insoluble in chloroform, and of intensely bitter taste. It was also found² to possess a characteristic action on the heart, closely resembling that of digitalis and the strophanthins. On the basis of his analyses and molecular weight determinations, Lehmann derived the formula $C_{30}H_{48}O_{12}$ for periplocin. The glucosidic character of the substance was shown by its cleavage by acid into reducing sugar and a bitter aglucone, *periplogenin*, of neutral character, which was obtained in crystalline form. Lehmann's analysis of the latter indicated $C_{24}H_{34}O_5$ as its formula. The sugar was obtained also in crystalline form but was not analyzed. His attempts to prepare an osazone were inconclusive, and the suggestion was offered that the sugar is a hexose, probably closely allied to glucose. The cleavage of periplocin by acid was given the following, as we shall see, improbable description:



Our own interest in periplocin began with our studies on the structural correlation of the aglucones of the cardiac glucosides

¹ Lehmann, E., *Arch. Pharm.*, 1897, cccxxv, 157.

² Burchinsky, P., *Russkij Wratsch.*, 1896, Nos. 29-35; *Petersburg med. Woch.*, 1897, No. 9, 49.

with strophanthidin. Through the kind cooperation of E. Merck and Company, Darmstadt, we obtained a small quantity of periplocin-Merck. This substance as described by Merck³ was a yellow amorphous powder soluble in water and alcohol. Following a few unpromising preliminary trials, no extensive attempts were made to isolate from the limited material at our disposal the crystalline glucoside of Lehmann, although Feigl⁴ described the separation of the crystalline glucoside from material obtained from Merck 20 years previously. Likewise our preliminary attempts to prepare the aglucone, periplogenin, directly from this material were not satisfactory. The observation was then made that the Keller-Kiliani reaction characteristic for desoxy sugars was given by the substance and that only a small amount could be extracted by chloroform from its aqueous solution. It was then found that the method used by us for the successful investigation of K-strophanthin⁵ could be employed here.

An aqueous solution of the substance on digestion with strophanthobiase prepared from *Strophanthus courmonti* seeds gave rise to a cleavage product which could now be readily extracted with chloroform. This substance crystallized readily under methyl alcohol with 1 mol of methyl alcohol of crystallization and in melting point and general properties resembled cymarin very closely. But further investigation showed definite differences. The analytical results agreed best with the formula $C_{30}H_{46}O_8 \cdot CH_3OH$, whereas the formula of cymarin is $C_{30}H_{44}O_9 \cdot CH_3OH$.

Although periplocin-Merck is described as a substance prepared from *Periploca græca*, in order to remove all uncertainty regarding the botanical origin of the material studied by us we have obtained from two other sources the plant itself with which careful comparative studies were made. Through the very generous cooperation of the late Professor Charles S. Sargent and of Dr. E. H. Wilson, we have received prunings of *Periploca græca* from the Arnold Arboretum. Likewise, through the kind efforts of Professor N. Maximow, Leningrad, material was collected for us by the staff of the Caucasus Division of the Institut de botanique appliquée et d'amélioration de plantes in Ssouchum. Of all of these kindnesses we are very deeply appreciative.

³ Merck, E., *Wissensch. Abhandl. Geb. Pharm.*, No. 8, 78.

⁴ Feigl, J., *Biochem Z.*, 1907, ii, 404.

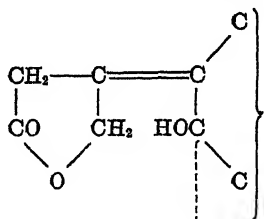
⁵ Jacobs, W. A., and Hoffmann, A., *J. Biol. Chem.*, 1926, lxi, 153.

Concentrated alcoholic extracts of the plant materials after preliminary purification when subjected in aqueous solution to the action of strophanthobiasse yielded a substance in each case which could be extracted with chloroform and which crystallized from methyl alcohol. These substances proved to be identical in all respects with that first obtained from the Merck product. *Periplocymarin*, as we have called the substance, is a glucoside. Like cymarin it gives the strong typical Keller-Kiliani reaction for desoxy sugars. In addition after removal of its solvent of crystallization, it still contains one methoxyl group which was found to belong to the sugar. Like cymarin and other glucosides of the desoxy sugars it is readily hydrolyzed by acid to the aglucone periplogenin and a methyl ether sugar. Although the latter was obtained as a low melting crystalline substance the limited material at our disposal prevented its preparation for analysis and possible identification with cymarose, $C_7H_{14}O_4$. This will be attempted when more material is available.

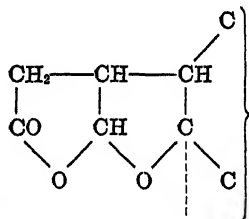
On the other hand, the aglucone was readily obtained in the crystalline form described by Lehmann for his substance. The analyses of our periplogenin were in best agreement with the formula $C_{23}H_{34}O_5$ as required for the above formulation of periplocymarin. This result differs from $C_{24}H_{34}O_5$, the formula adopted by Lehmann. However, as we shall see, our conclusion has been confirmed by the analysis of a number of the derivatives of the substance. Periplogenin is isomeric with gitoxigenin which we have found to possess also the formula $C_{23}H_{34}O_5$. Although neutral, periplogenin when boiled with alkali takes up 1 equivalent due to the saponification of a lactone group. On catalytic hydrogenation it absorbs 1 mol of hydrogen with the formation of *dihydropерiplogenin*. Periplogenin is, therefore, an unsaturated lactone. The double bond is associated with the lactone group since it gives the Legal reaction and its dihydro derivative no longer reacts with nitroprusside. Periplogenin does not combine with ketone reagents. It possesses three hydroxyl groups of which only one can be directly benzoylated.

Of great importance are the results obtained in the study of the action of alkali on periplogenin. In methyl alcoholic alkali periplogenin is isomerized to an iso compound, *isoperiplogenin*, in a manner exactly analogous to that ascertained in the case of

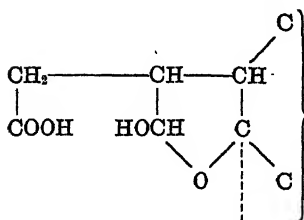
strophanthidin and digitoxigenin. The beautifully crystallizing iso derivative proved to be structurally analogous to isostrophanthidin⁶ and isodigitoxigenin.⁷ Like these substances, it no longer gives the nitroprusside reaction and cannot be hydrogenated. On saponification it yields a hydroxyaldehyde acid. This hydroxyaldehyde acid in solution as a salt was oxidized by sodium hypobromite directly in the lactol form to the salt of a lactone acid, *isoperiplogenic acid*, $C_{23}H_{34}O_6$, which was isolated in beautifully crystallizing form. This formulation was confirmed by its titration and the analysis of its *methyl ester*. These results permit the conclusion that periplogenin like strophanthidin, digitoxigenin, and gitoxigenin is a $\Delta^{\beta, \gamma}$ -lactone possessing 23 carbon atoms and is composed of 4 saturated rings. The lactone group consists of a side chain which is attached by its β -carbon atom to a ring containing a hydroxyl group, presumably tertiary in character. This hydroxyl is attached to a carbon atom probably γ to the γ -carbon of the lactone ring, as shown in the partial Formula I.



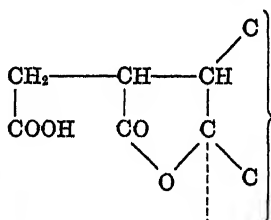
I.



II.



III.



IV.

Under the influence of alkali the iso compound is formed with the formation of a new ring as shown in Formula II. On saponi-

⁶ Jacobs, W. A., and Gustus, E. L., *J. Biol. Chem.*, 1927, lxxiv, 811.

⁷ Jacobs, W. A., and Gustus, E. L., *J. Biol. Chem.*, 1928, lxxviii, 573.

fication this gives an acid which may exist in the lactol form, Formula III, and this in turn may be oxidized directly to the lactone acid (Formula IV).

Owing to the very limited amount of material at our disposal, we have not been able to complete all of the steps which were studied in the case of isostrophanthidin and isodigitoxigenin. The preparation of an oxime from the ester of isoperiplogenic acid and other transformations must await the acquisition of more material. We have carried through a series of reactions in which isoperiplogenin was saponified to isoperiplogenic acid, the latter esterified with diazo methane, and the resulting methyl ester oxidized with chromic acid to a ketolactone ester. The attempt was made to isolate only the last substance but unfortunately it was obtained in too small an amount to permit of satisfactory purification for analysis. We hope to be in a position to return to these substances as well as to the general problem of the further correlation of periplogenin with strophanthidin and the digitalis aglucones. Finally, an attempt is being made to procure more plant material in order to prepare the crystalline periplocin of Lehmann for further study. It appears probable that this substance is a polysaccharide glucoside of periplogenin analogous to K-strophanthin- β , which may be cleaved by the enzyme to glucose and periplocymarin. The fact that Lehmann, after acid hydrolysis, was unable to prepare an osazone is suggestive of the similar experience which we have described in the case of K-strophanthin- β . And finally, the analytical figures reported by him for periplocin are in accordance with this view, especially if the assumption is made that the substance analyzed by him retained 1 mol of water. This may be seen from the following.

Lehmann's analysis. C 60.42, H 8.4.
C₃₆H₅₆O₁₃. " 62.03, " 8.10.
C₃₆H₅₆O₁₃·H₂O. " 60.46, " 8.18.

EXPERIMENTAL.

Periplocymarin.—The thick stems and branches of *Periploca græca* still containing the bark were received in a dry condition from Ssouchum (Black Sea district), Russia. This material was ground in a drug mill to a fine powder.

6.5 kilos of this powder, which was mostly cellulose, were extracted several times with 20 liters of 70 per cent alcohol. The extracts were precipitated with basic lead acetate and then filtered from an appreciable amorphous precipitate. The filtrates were treated with an excess of sodium phosphate to remove the dissolved lead and were again filtered. The combined filtrates were then concentrated under diminished pressure to remove the alcohol. During this process, which was made difficult by foaming, appreciable amounts of resinous material separated, and when the concentration was carried to a certain point a gum also began to deposit. At this point the mixture was extracted with ether in order to remove the resinous material. The aqueous solution was then further concentrated to about 1500 cc., causing the deposition of an appreciable amount of gum. The mixture was extracted several times with chloroform. During this time the gum remained as an intermediate layer between the chloroform and aqueous layers. The chloroform extract after concentration yielded a resinous residue which gave only a weak Keller-Kiliani reaction.

The aqueous layer was decanted from the gum and because of appreciable acidity it was brought to a pH of about 6.0 with sodium carbonate solution. A concentrated solution of 2.5 gm. of strophanthobias prepared from *Strophanthus courmonti* seeds was then added, followed by chloroform for preservation, and the mixture was kept at 38° for 7 days. The mixture was directly extracted four or five times with chloroform. In the course of this process rather persistent emulsions were formed.

The chloroform extract was washed with a little dilute sodium carbonate solution which removed some light brown coloring matter. The dried chloroform extract after concentration to smaller volume gave, with petroleic ether, a colorless precipitate. After collection this was found to crystallize readily under methyl alcohol. After collection with methyl alcohol, 1 gm. of periplocymarin was obtained.

The above aqueous enzyme solution after extraction was treated again with 2.5 gm. of enzyme, and after standing for 2 weeks at 38° was extracted with chloroform as before, and by the procedure previously used gave an additional 0.6 gm. of crystalline glucoside. The enzyme solution was then returned to the thermostat

and after 3 months yielded 1.3 gm. more. The total yield was 2.9 gm.

Recrystallized from methyl alcohol, periplocymarin separates as lustrous needles which contain approximately 1 mol of methyl alcohol; this is partly lost on air-drying and completely lost on drying at 100° under diminished pressure. It sinters at about 138° and melts at 148°. In melting point, general appearance, and solubilities it resembles cymarin very closely. The alcoholic solution of periplocymarin is decidedly bitter but the taste is not nearly so marked as that of cymarin. It is readily soluble in chloroform, acetone, and ethyl alcohol and less readily in methyl alcohol. It is very sparingly soluble in water and practically insoluble in ether. Due to the presence of a desoxy sugar the glucoside gives the characteristic Keller-Kiliani reaction. It is, therefore, readily hydrolyzed in the cold to the aglucone and the sugar.

$[\alpha]_D^{27} = +29$ ($c = 0.943$ in 95 per cent alcohol for the anhydrous substance).

For analysis the substance was dried at 100° and 20 mm.

Air-Dry Substance.

4.282 mg. substance: 3.181 mg. Ag I.

$C_{30}H_{46}O_8 \cdot CH_3OH$. Calculated. OCH_3 , 10.94. Found. 9.80.

Dried Substance.

3.307 mg. substance: 2.550 mg. H_2O , 8.134 mg. CO_2 .

3.897 " " : 3.140 " " 9.622 " "

4.070 " " : 1.748 " Ag I.

$C_{30}H_{46}O_8$. Calculated. C 67.37, H 8.68, OCH_3 5.80.

Found. " 67.08, " 8.63.

" " 67.33, " 9.00.

"

OCH_3 5.67.

400 gm. of fresh *Periploca græca* stems and branches obtained from the Harvard Arboretum, when worked up with enzyme in the manner previously described, yielded 0.2 gm. of periplocymarin which agreed in all properties with the substance described above.

Periplocin-Merck, which was used for the following experiments, formed a light tan-colored powder in which no crystalline structure could be detected, and all preliminary attempts with the limited amount of material at our disposal failed to give by direct methods a crystalline product. The substance was obviously a complex mixture, as the following experiment showed.

1 gm. of amorphous periplocin-Merck was dissolved in 100 cc. of water. When extraction of the solution with chloroform was attempted, a tenacious emulsion formed. However, when a solution of 0.2 gm. of strophanthobiase in 2 cc. of water was added, the emulsion rapidly cleared and the aqueous solution could be readily extracted with chloroform. The extraction was repeated several times. The washed and dried chloroform extracts yielded on concentration a resinous residue of 0.1 gm. which could not be made to crystallize and which gave a weak, atypical Keller-Kiliani reaction.

The remaining aqueous fraction containing the enzyme was allowed to stand 7 days at 38° and was then extracted with chloroform as in the previous cases. The residue from the chloroform readily crystallized in characteristic form under methyl alcohol and yielded 0.17 gm. of needles which sintered above 135° and melted at 145–148°. This material proved to be identical in all respects with the periplocymarin obtained from the other sources. In a second experiment in which 5 gm. of periplocin-Merck were digested with enzyme, 1.4 gm. of amorphous substance was obtained by precipitation of the concentrated chloroform extracts with petroleum ether. This material gave 0.8 gm. of pure substance when it was twice recrystallized from methyl alcohol.

For analysis, the substance was dried at 100° and 20 mm.

0.1003 gm. substance:	0.0803 gm. H ₂ O,	0.2462 gm. CO ₂ .
0.1001 " " :	0.0795 " " :	0.2465 " "
0.0831 " " :	0.0359 " Ag I.	
C ₃₀ H ₄₆ O ₈ .	Calculated.	C 67.37, H 8.68, OCH ₃ 5.80.
	Found.	" 66.95, " 8.96.
	"	" 67.16, " 8.89.
	"	OCH ₃ 5.71.

Periplogenin.—0.9 gm. of periplocymarin (from the *Ssouchum* plant) was dissolved in 9 cc. of alcohol and the solution was treated with 18 cc. of 10 per cent hydrochloric acid. The mixture was then left at room temperature for 4 hours. On dilution and rubbing the "genin" slowly crystallized as plates and long prisms. After standing at 0° overnight, the substance was collected with water. 0.45 gm. was directly obtained. The mother liquor to which a little alcohol was added to hold in solution the portion of aglucone still dissolved was treated with silver carbonate and

filtered. The filtrate was freed from traces of silver by hydrogen sulfide, then cleared with bone-black, and finally concentrated under diminished pressure. The residue when treated with a small volume of water yielded an additional 0.1 gm. of periplogenin, a total of 0.55 gm. or 90 per cent of the theory.

Upon recrystallization by careful dilution of its alcoholic solution, periplogenin separates as small prisms which contain when air-dry about 1.5 mols of H_2O . It melts with effervescence when rapidly heated at $135-140^\circ$. The melting point, however, varied with different preparations and appeared to depend upon the form in which it crystallized. On several occasions when it was recrystallized from dilute alcohol a melting point of $168-169^\circ$ was observed. When recrystallized from methyl alcohol, it formed stout prisms which sintered at 140° but did not melt until 235° and contained methyl alcohol of crystallization. No attempt was made to investigate the causes of the variable melting points, an experience which we have frequently encountered with strophanthidin and its derivatives. For the periplogenin which he described, Lehmann reported a melting point of 185° . The behavior of the periplogenin samples from our three different sources towards sulfuric acid also differed somewhat from that described by Lehmann for his substance. According to the latter, periplogenin crystals are colored blue at once when covered with sulfuric acid; then as the crystals dissolve the color changes to rose and finally disappears. All of our substances, when covered on a watch-glass with sulfuric acid, dissolved at once with an orange-yellow or bright golden color. When the mixture stood exposed to the air, a deep indigo blue developed around the rim, finally extending through the whole solution.

The solubilities and rotations were found to be as reported by Lehmann. $[\alpha]_D^{25} = +31.5^\circ$ ($c = 1.04$ in alcohol). Lehmann found $[\alpha] = +30^\circ$ in alcohol.

Periplogenin does not contain a methoxyl group, and although a neutral substance it consumes 1 equivalent on boiling with alkali, due to the lactone group. It gives a strong, positive Legal test. In an attempt to prepare an oxime, periplogenin was recovered unchanged.

For analysis, the air-dried substance was dried at 100° and 20 mm.

2.999 mg. substance: 0.193 mg. H_2O .

$\text{C}_{23}\text{H}_{34}\text{O}_5 \cdot 1\frac{1}{2} \text{H}_2\text{O}$. Calculated. H_2O 6.47. Found. H_2O 6.50.

2.922 mg. substance: 2.242 mg. H_2O , 7.590 mg. CO_2 .

2.806 " " : 2.231 " " 7.292 " "

$\text{C}_{23}\text{H}_{34}\text{O}_5$. Calculated. C 70.72, H 8.78.

Found. " 70.83, " 8.58.

" " 70.86, " 8.88.

For the titration, 14.7 mg. of substance were refluxed for 2 hours with 0.5 cc. of alcohol and 0.80 cc. of 0.1 N NaOH and titrated back against phenolphthalein. Calculated for 1 equivalent, 0.38 cc. Found, 0.39 cc.

Periplogenin Benzoate.—In pyridine solution with benzoylchloride, a monobenzoate was readily obtained. When it was recrystallized from 95 per cent alcohol, it formed sparingly soluble, stout, glistening wedges which lost nothing when heated at 100° and 20 mm. When it was rapidly heated, the substance melted at 235° , although this was found to depend upon the rate of heating.

3.917 mg. substance: 2.800 mg. H_2O , 10.428 mg. CO_2 .

$\text{C}_{30}\text{H}_{38}\text{O}_6$. Calculated. C 72.83, H 7.75.

Found. " 72.60, " 8.00.

Dihydroperiplogenin.—Periplogenin after careful recrystallization from glass-distilled solvents was hydrogenated with an active platinum black. 0.15 gm. of substance absorbed within an hour 10 cc. or 1 mol of H_2 . The absorption then stopped. The filtrate on evaporation readily yielded the dihydro compound as flat prisms, plates, or broad, stout needles which were collected with 25 per cent alcohol. After recrystallization from dilute alcohol it melted at 204° after slight preliminary softening. The substance is easily soluble in alcohol, acetone, and chloroform and very sparingly soluble in ether and water. It gives no color reaction with sodium nitroprusside.

For analysis the substance was dried at 100° and 20 mm.

3.174 mg. substance: 2.583 mg. H_2O , 8.202 mg. CO_2 .

4.660 " " : 3.975 " " 12.060 " "

$\text{C}_{23}\text{H}_{34}\text{O}_5$. Calculated. C 70.36, H 9.25.

Found. " 70.47, " 9.10.

" " 70.57, " 9.54.

Isoperiplogenin.—Periplogenin was shaken in 30 parts of methyl alcoholic sodium hydroxide (2 gm.: 100 cc.) to facilitate solution. After several hours the solution was carefully neutralized with acetic acid and concentrated to remove the alcohol. The sodium salt of the iso acid which was formed by saponification remained as needles and was easily soluble in water. When the solution was acidified to Congo red with hydrochloric acid an amorphous precipitate formed, which gradually crystallized. Recrystallized from alcohol, isoperiplogenin formed practically anhydrous leaflets which melted at 258° after preliminary softening. From dilute alcohol the substance separates as broad platelets and bundles of needles or long, narrow leaflets which melted at 248°. The substance is soluble in chloroform, acetone, and alcohol and is practically insoluble in ether and water. It does not give the nitroprusside test. Its behavior with sulfuric acid is very characteristic. When covered on a watch-glass with this reagent, it dissolves at once, forming a deep claret-colored solution. Around the edges a blue color slowly develops, as in the case of periplogenin, and finally extends through the solution.

2.820 mg. substance: 2.244 mg. H_2O , 7.323 mg. CO_2 .

$C_{22}H_{34}O_5$. Calculated. C 70.72, H 8.78.

Found. " 70.81, " 8.56.

Isoperiplogenic Acid.—0.1 gm. of isoperiplogenin was covered with 0.5 cc. of pyridine and 2 cc. of 0.5 N NaOH and shaken until dissolved due to saponification. The mixture was carefully neutralized with acetic acid and then treated with 1 cc. of a solution of bromine in N NaOH containing 60 mg. per cc. After it had stood for 1 hour, acidification with acetic acid caused the prompt precipitation of the crystalline lactone acid. After collection with water it was recrystallized by careful dilution of its alcoholic solution. It formed lustrous six-sided leaflets which contained water of crystallization. The substance melts with effervescence at 215°. It is soluble in alcohol and acetone and but sparingly soluble in chloroform. In ether and water it is practically insoluble. In sulfuric acid it forms a deep amber-colored solution.

The substance as above obtained separates apparently with 1 mol of water of crystallization which it partly loses on standing in a desiccator. However, after it was attempted to remove the

water entirely by drying at 100° and 20 mm., the analytical results obtained with several preparations indicated the retention of 0.5 mol of water in the dried samples.

Sample A.	4.056 mg. substance:	3.102 mg. H ₂ O,	9.870 mg. CO ₂ .
" B.	3.384 "	" : 2.650 "	" 8.214 " "
C ₂₃ H ₃₄ O ₆ .	Calculated.	C 67.99,	H 8.44.
C ₂₃ H ₃₄ O ₆ · ½ H ₂ O.	"	" 66.46,	" 8.50.
	Found. Sample A.	" 66.36,	" 8.56.
	" B.	" 66.20,	" 8.76.

10.834 mg. of dried substance were treated with 1 cc. of alcohol and titrated directly against phenolphthalein with 0.1 N NaOH. Calculated for 1 equivalent for C₂₃H₃₄O₆ · ½ H₂O, 0.261 cc. Found, 0.266 cc.

3 cc. additional of 0.1 N NaOH were then added and after being refluxed for 4 hours the solution was titrated back. Calculated for 1 equivalent, 0.261 cc. Found, 0.267 cc.

On acidification of the above titration solution to Congo red the lactone acid again slowly crystallized after lactonization occurred.

Isoperiplogenic Methyl Ester.—The above acid, when treated with diazo methane in acetone solution, readily yielded the ester which crystallized from acetone as long prisms and needles which melted at 252°. The ester is soluble in descending order in chloroform, acetone, and methyl alcohol and practically insoluble in ether. Correct analytical figures were obtained with this derivative.

3.546 mg. substance:	2.782 mg. H ₂ O,	8.883 mg. CO ₂ .
3.582 " "	: 2.794 " "	8.990 " "
C ₂₄ H ₃₆ O ₆ .	Calculated.	C 68.53, H 8.64.
	Found.	" 68.31, " 8.78.
	"	" 68.44, " 8.73.

STROPHANTHIN.

XV. HISPIDUS STROPHANTHIN.

BY WALTER A. JACOBS AND ALEXANDER HOFFMANN.

(From the Laboratories of The Rockefeller Institute for Medical Research,
New York.)

(Received for publication, June 25, 1928.)

The work of Heffter and Sachs¹ and that of Brauns and Closson² have shown that the strophanthin obtained from *Strophanthus hispidus* seeds is a strophanthidin derivative like the strophanthins which occur in the seeds of *Strophanthus kombe*. The former workers reached the conclusion from a comparison of the so called amorphous strophanthins from both plants that they are identical or certainly very closely related. It was noted, however, by both groups of workers that a crystalline strophanthin could not be obtained from *hispidus* seeds as in the case of the *kombe* variety. Finally, all attempts to ascertain the nature of the sugar in the strophanthins from both sources were unsuccessful and in no case could an osazone be obtained.

In previous communications,³ we have shown that the so called "crystalline K-strophanthin" is a mixture of cymarín, glucosidocymarín (K-strophanthin- β), and amorphous polyglucosidocymaríns, and that the so called "amorphous K-strophanthin" is likewise a mixture of glucose cymarín derivatives which could be hydrolyzed to glucose and cymarín by means of strophanthobiase, an enzyme prepared from *Strophanthus (courmonti)* seeds.

Since no crystalline strophanthin had been obtained from *hispidus* seeds by previous workers, the question was raised in our minds as to the identity of the strophanthins from both sources,

¹ Heffter, A., and Sachs, F., *Biochem. Z.*, 1912, xl, 83.

² Brauns, D. H., and Closson, O. E., *J. Am. Pharm. Assn.*, 1913, ii, 489, 604, 715.

³ Jacobs, W. A., and Hoffmann, A., *J. Biol. Chem.*, 1926, lxxvii, 609; 1926, lxxix, 153.

at least in respect to the carbohydrate portion of the molecule. It was hoped, therefore, that the method which permitted the complete characterization of the strophanthins occurring in *Strophanthus kombe* seeds might be applied also in this case.

Through the very generous cooperation of Drs. Wilbur A. Sawyer and Henry Hanson of the International Health Division of the Rockefeller Foundation and Mr. J. R. Ainslie, Senior Conservator of Forests, Ibadan, Nigeria, we have received several batches of *Strophanthus hispidus* seeds of certain identity, with which the present investigations have been made.

The seeds were ground and defatted with ligroin. 800 gm. of this powder were extracted four times with 70 per cent alcohol at room temperature. The combined extracts were precipitated with basic lead acetate and the excess lead was then removed from the filtrate by ammonium sulfate solution. Concentration of the clear amber-colored filtrate to about 1 liter caused the deposition of a small amount of oily resin which was removed by repeated extraction of the mixture with ether. The aqueous solution was then extracted three times with about 100 cc. of chloroform. The chloroform extract was washed with a little dilute sodium carbonate solution, dried, and then concentrated to small bulk. Petrolie ether gave a copious, amorphous, flocculent precipitate which after collection weighed 3.4 gm. This material, which gave a strong Keller-Kiliani reaction, was dissolved in 10 cc. of methyl alcohol. After it was seeded with cymarín and kept at 0° for several days, a slow crystalline crust separated. This was collected with cold methyl alcohol. 0.7 gm. of substance was obtained.

After recrystallization from methyl alcohol it formed stout, short, thick prisms which melted with slow effervescence at 147–153°. The substance gave a strong Keller-Kiliani desoxy sugar reaction and proved to be identical in all respects with cymarín. $[\alpha]_D^{25} = +36^\circ$ ($c = 0.945$ in 95 per cent alcohol for the dried substance).

Air-Dry Substance.—Dried at 100° and 20 mm.

2.628 mg. substance: 2.016 mg. AgI.

$C_{30}H_{44}O_9 \cdot CH_3OH$. Calculated. $2(OCH_3)$, 10.69. Found. (OCH_3) , 10.12.
“ CH_3OH , 5.52. Found, 5.20.

Dry Substance.

3.237 mg. substance: 2.367 mg. H_2O , 7.745 mg. CO_2 .

$C_{30}H_{44}O_8$. Calculated. C 65.66, H 8.09.

Found. " 65.24, " 8.18.

When it was hydrolyzed in the usual way, strophanthidin was obtained in characteristic form and melting point, 177–178°.

3.068 mg. substance: 2.255 mg. H_2O , 7.505 mg. CO_2 .

$C_{23}H_{32}O_6 \cdot \frac{1}{2} H_2O$. Calculated. C 66.79, H 8.05.

Found. " 66.70, " 8.22.

The mother liquor from the 0.7 gm. of cymarín yielded very little more crystalline cymarín on further manipulation since most of the material was of undetermined, non-crystalline character.

From the aqueous solution containing the chloroform-insoluble glucosides, the chloroform which adhered to the solution after the extraction process was removed by suction and the remaining clear aqueous solution was saturated with ammonium sulfate. A copious gummy precipitate of strophanthin formed, which slowly hardened to a resin. The supernatant solution was decanted and the resin was covered with absolute alcohol. The strophanthin gradually dissolved, leaving crystals of ammonium sulfate which were sucked off and washed with alcohol. The filtrate after concentration to dryness at 20 mm. left a light amber-colored gum which was again dissolved in absolute alcohol, leaving a small amount of resinous material and ammonium sulfate. The filtrate was again concentrated and the residue was dissolved in 400 cc. of water. This solution contained no free reducing sugars.

100 cc., or one-quarter of this solution, were treated with acid and hydrolyzed in the usual manner for strophanthidin. This was obtained in copious amount and after recrystallization weighed 2.8 gm. It melted at 176–177° with effervescence and proved to be identical in all respects with strophanthidin.

3.805 mg. substance: 2.870 mg. H_2O , 9.315 mg. CO_2 .

$C_{23}H_{32}O_6 \cdot \frac{1}{2} H_2O$. Calculated. C 66.79, H 8.05.

Found. " 66.83, " 8.43.

The remainder (300 cc.) of the aqueous solution of the amorphous strophanthin, which was somewhat acid, was carefully

neutralized to litmus. The solution was treated with 0.5 gm. of strophanthobiase obtained from *Strophanthus courmonti* seeds and which exhibited a strong action on K-strophanthin. After 10 days at 38°, the solution gave a reduction with Fehling's solution equivalent to 6.7 mg. of glucose per cc. Since this did not increase appreciably after 3 days, the mixture was directly extracted a number of times with chloroform. The dried and concentrated chloroform extract gave with ligroin an amorphous precipitate which after collection weighed 1.7 gm. This material gave a very weak Keller-Kiliani reaction and from it no crystalline cymarins could be obtained. The aqueous solution containing the unaffected glucosides and the sugar, which had been cleaved by the enzyme, were precipitated with ammonium sulfate and the supernatant solution was decanted from the copious gummy strophanthin. The decanted solution was poured into a large volume of alcohol to precipitate the salt and the filtrate was concentrated to dryness. From the residual sugar syrup 0.2 gm. of an osazone was obtained. This melted at 210° and exhibited the following optical behavior.⁴ 0.1 gm. dissolved in a mixture of 2 cc. of pyridine and 3 cc. of alcohol in a 0.5 dm. tube showed $\alpha_D = -0.67^\circ$ immediately after solution. After 19 hours $\alpha_D = -0.38^\circ$. The substance was, therefore, *d*-glucosazone.

2.944 mg. substance: 1.730 mg. H₂O, 6.488 mg. CO₂.

C₁₃H₂₂O₄N₄. Calculated. C 60.30, H 6.19.

Found. " 60.13, " 6.56.

The strophanthin gum was dissolved again in alcohol and treated in the manner previously described in order to remove the ammonium sulfate used for its precipitation and was finally brought to a solution in 250 cc. of water. This solution showed a reducing action for Fehling's solution corresponding to 0.85 mg. of glucose per cc.

An enzyme preparation from *Strophanthus hispidus* seeds was then made as follows. 100 gm. of *Strophanthus hispidus* seeds, after being ground and defatted, were extracted with 500 cc. of thymol water. The filtrate was precipitated with 4 volumes of

⁴ Levene, P. A., and La Forge, F. B., *J. Biol. Chem.*, 1915, xx, 429.

alcohol and after being allowed to settle was decanted. The precipitate was treated several times with alcohol and was finally collected with absolute alcohol and dried thoroughly over calcium chloride. This material was used as such by dissolving it as needed in about 5 parts of water. The enzyme proved to be very active towards K-strophanthin- β , since 0.1 gm. of the enzyme caused practically complete cleavage of 0.2 gm. of the glucoside into glucose and cymarin in 24 to 48 hours.

200 cc. of the strophanthin solution were treated with a solution of 1 gm. of *hispidus* enzyme and digested at 38°. After 7 days the solution gave a reduction corresponding to 18 mg. of glucose per cc. After 11 days this increased to 19 mg. per cc. The solution was then treated with 4 volumes of alcohol to coagulate the enzyme and after being filtered through bone-black the alcohol was distilled off. The aqueous solution was extracted several times with chloroform. During this process, an intermediate layer of resinous material formed, which had not been observed before the action of the enzyme. This layer redissolved on further dilution with water. The chloroform extract, after being washed, dried, and concentrated, gave an appreciable precipitate with ligroin. The yield was 1.6 gm. Upon dissolving in a few cc. of methyl alcohol, it partly crystallized when it was seeded with cymarin in characteristic form. After standing in the refrigerator, this was collected with methyl alcohol. The yield was 0.5 gm.

After recrystallization from methyl alcohol, the cymarin crystallized in the usual form as prisms and needles which melted and effervesced at 145–146° after preliminary softening.

Air-Dry Substance.—Dried at 100° and 20 mm.

4.045 mg. substance: 0.235 mg. CH_3OH .

$\text{C}_{30}\text{H}_{44}\text{O}_9 \cdot \text{CH}_3\text{OH}$. Calculated. CH_3OH 5.52. Found. CH_3OH 5.81.

Anhydrous Substance.

3.810 mg. substance: 2.871 mg. H_2O , 9.177 mg. CO_2 .

$\text{C}_{30}\text{H}_{44}\text{O}_9$. Calculated. C 65.66, H 8.09.

Found. " 65.68, " 8.43.

The mother liquor from the cymarin on evaporation dried to a glass which could not be crystallized.

The above aqueous solution, which remained after extraction with chloroform of the cymarin liberated by the enzyme, was freed from adhering chloroform and was precipitated with am-

monium sulfate. A copious precipitate of gummy strophanthin was again formed. The supernatant liquor containing the free sugar was decanted. The latter was mixed with several volumes of alcohol, filtered from ammonium sulfate, and was then concentrated to a syrup. The syrup was again dissolved in 85 per cent alcohol and was then filtered from further amounts of ammonium sulfate. The alcoholic filtrate on concentration left the sugar solution which was made up to 50 cc. with water. 1 cc. of this gave a reduction with Fehling's solution which indicated 0.0404 gm. per cc. for glucose. $\alpha_D = +2.14^\circ$ or $[\alpha]_D = +53^\circ$ ($c = 4.04$). For glucose, $[\alpha]_D = +52.5^\circ$.

That the sugar is, therefore, glucose was confirmed by the preparation of the phenylosazone in the usual manner. After recrystallization from 70 per cent alcohol this melted at $210-211^\circ$. In its optical behavior the osazone also showed that required for phenylglucosazone. For the solution of 0.1 gm. in a mixture of 2 cc. of pyridine and 3 cc. of alcohol in a 1 dm. tube $\alpha_D = -1.30^\circ$ immediately after solution. After 24 hours $\alpha_D = -0.68^\circ$.

3.370 mg. substance: 1.912 mg. H_2O , 7.430 mg. CO_2 .

$C_{13}H_{22}O_4N_4$. Calculated. C 60.30, H 6.19.

Found. " 60.12, " 6.35.

The amorphous gummy strophanthin recovered from the enzyme experiment was separated from ammonium sulfate as previously described. A solution in 100 cc. of water was then made with the recovered strophanthin and subjected again to the action of 1 gm. of fresh *hispidus* enzyme. After 1 week the mixture was extracted with chloroform. This yielded 0.3 gm. of recrystallized cymarín along with non-crystallizable resinous material. Most of the strophanthin remained, however, in the aqueous layer and could be recovered with ammonium sulfate.

Several parallel attempts to cleave the amorphous strophanthin with strophanthobiase from *Strophanthus courmonti* failed to yield any crystalline cymarín although in several instances evidence of slight cleavage of sugar was noted.

From these experiments the following conclusions may be drawn. In confirmation of Heffter and Sachs and Brauns and Closson, the aglucone of *hispidus* strophanthin is identical with the strophanthidin of the *kombe* strophanthins. *Hispidus*

strophanthin like *kombe* strophanthin is a mixture of glucosides. Among these cymarin occurs both free and combined with glucose but here the analogy stops. The nature of the union between cymarin and glucose may be different from that in which it occurs in *kombe* strophanthin, since its cleavage can be accomplished only slowly and incompletely by means of the special enzymes occurring in *hispidus* seeds. Whether other sugars or substances enter into the make-up of these glucosides and cause this relative resistance to enzyme cleavage has not been determined. Where cleavage has occurred, however, only glucose and cymarin have been identified. *Hispidus* strophanthin is, therefore, chemically different from K-strophanthin. This fact should be recognized and considered before the pharmacological equivalence of the strophanthin mixtures obtained from both sources is assumed.

STROPHANTHIN.

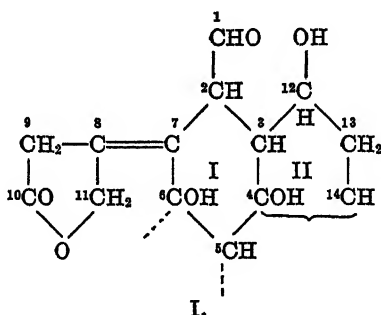
XVI. DEGRADATION IN THE ISOSTROPHANTHIDIN SERIES.

By WALTER A. JACOBS AND EDWIN L. GUSTUS.

(From the Laboratories of The Rockefeller Institute for Medical Research,
New York.)

(Received for publication, July 3, 1928.)

The interrelationships as regards allocation of the three hydroxyl groups, the aldehyde group, and the unsaturated lactone group of the strophanthidin molecule have been deduced by methods presented in previous studies in the strophanthidin and isostrophanthidin series.¹ These relationships are represented in the accompanying graphic formula in which we have now adopted numerical designations for the individual carbon atoms and rings.



Since recent work has shown that substances such as periplogenin² and digitoxigenin,³ which do not possess free carbonyl groups, may nevertheless be isomerized to iso compounds, it now appears that the assumption is not necessary that enolization of

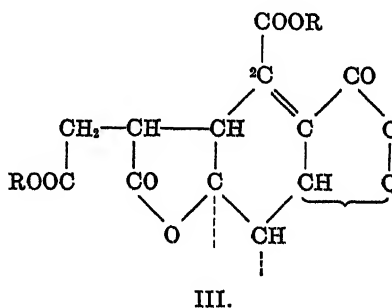
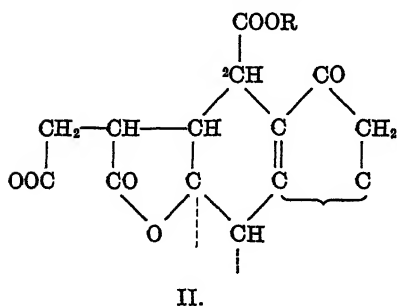
¹ Jacobs, W. A., and Gustus, E. L., *J. Biol. Chem.*, 1927, lxxiv, 795, 805, 811, 829.

² Jacobs, W. A., and Hoffmann, A., *J. Biol. Chem.*, 1928, lxxix, 519.

³ Jacobs, W. A., and Gustus, E. L., *J. Biol. Chem.*, 1928, lxxviii, 573.

the aldehyde group is the determining factor in the shift of the double bond. Carbon atom 7 is, therefore, not the only point which may be considered as the place of attachment of the lactone group, which previous oxidation experiments with trianhydrostrophanthidin have definitely shown to be situated on a carbon atom of ring I. Carbon atom 5 may also be considered in this regard.

Although the relative positions of the hydroxyl groups previously adopted appear to rest on good evidence, it is nevertheless desirable to supplement this in other ways, especially by means of oxidative degradation, and to carry on more extensive structural studies by this method. For this purpose we have attempted to develop further the opening made in the previously reported oxidation experiments with trianhydrostrophanthidin. Unfortunately a continuation in this direction has been temporarily halted by the unpromising yields of the substances obtained, so that our attention has been directed to the isostrophanthidin series. The substance which appeared as a promising starting point is anhydro- α -isostrophanthonic acid⁴ or its ester, for which Formulæ II and III may be considered.

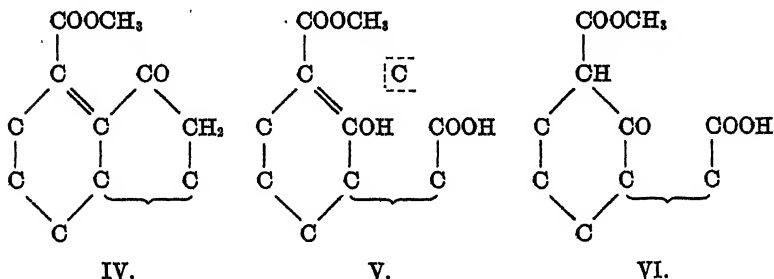


Since this substance does not give the Legal test, Formula III is to be preferred. In the first case the substance would be a $\Delta^{\beta,\gamma}$ -ester and the hydrogen attached to carbon atom 2 should be active and give the Legal reaction. The substance with Formula II, however, must be the precursor of anhydro- α -isostrophanthonic ester (III).

⁴ Jacobs, W. A., and Gustus, E. L., *J. Biol. Chem.*, 1927, lxxiv, 819.

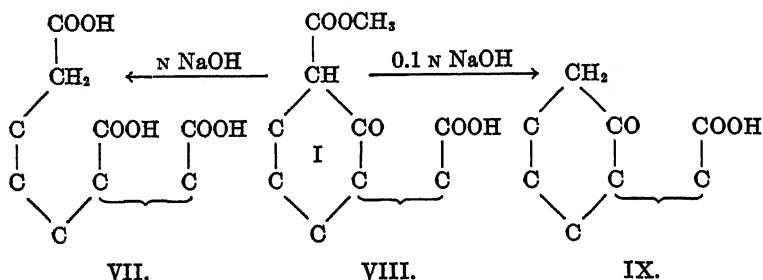
Both substance III and its parent compound, α -isostrophanthonic dimethyl ester, are unaffected under ordinary conditions by chromic acid, the reagent used for the preparation of the latter. On the other hand, a difference was noted in their behavior towards permanganate. Whereas the saturated substance proved to be quite resistant to the action of this reagent in acetone solution, the unsaturated derivative was rapidly oxidized. From the reaction mixture two acid products have been isolated which analyses have indicated to have the formulæ, $C_{24}H_{32}O_9$ and $C_{25}H_{32}O_{10}$, respectively. The first substance proved to be the dimethyl ester of a tribasic lactone acid and could be more conveniently purified after conversion into the neutral trimethyl ester, $C_{25}H_{34}O_9$. This oxidation product was also obtained as the monomethyl ester by the oxidation of the monomethyl ester of anhydro- α -isostrophanthonic acid in aqueous solution with permanganate.

The formation of this compound from the parent substance has obviously involved the rupture of ring II with the loss of 1 carbon atom. Investigation showed that it also possesses a carbonyl group and readily forms an oxime. The tribasic lactone acid has, therefore, been called *undephanthontriacid*. The neutral ester of this acid exhibited the characteristic behavior of a β -keto ester and its formation from anhydro- α -isostrophanthonic dimethyl ester must have involved the steps pictured in the accompanying partial formulæ (IV to VI).



A suggestive point has appeared in the difference in behavior towards 0.1 N alkali of the ester group of carbon atom 1 in the parent substance and of that in its oxidation product. As already noted⁴ the one ester group in anhydro- α -isostrophanthonic di-

methyl ester is relatively resistant to the action of this reagent. On the other hand in undephanthontriacid dimethyl ester, both ester groups are readily saponified. The relative stability in the former substance may be conditioned by the di-ortho substitution on either side of carboxyl 1. Ring cleavage at C³ (cf. Formula I) should tend to remove steric hindrance and render the oxidation product more easily saponified. Since the saponification product was a β -keto acid it was not isolated as such but after loss of CO₂ as the ketone *duodephanthondiacid* in which remain only 21 of the carbon atoms of the strophanthidin molecule (Formula IX). This substance was further characterized by the preparation of an oxime.



When N alkali was employed undephanthontriacid trimethyl ester underwent the acid decomposition (Formula VII), a fact which was shown by its consumption of 5 equivalents of alkali instead of 4. Unfortunately, the resulting tetrabasic lactone acid could not be isolated in crystalline form. The formation of this substance has involved the rupture of ring I, which is the first observation of this kind which has been made in our studies with strophanthidin.

Undephanthontriacid trimethyl ester formed not only an oxime but also a phenylhydrazone which showed the characteristic property of a β -keto ester derivative by forming when heated a phenyllactazam.

The second substance which was isolated in smaller yield from among the oxidation products of anhydro- α -isostrophanthonic dimethyl ester has been more difficult to characterize and its relationship to the parent substance is, therefore, obscure. Titration experiments showed it to be monobasic and that it possessed

the lactone group and the original two methyl ester groups. It did not appear to react with ketone reagents and could not be acylated. However, 2 active hydrogen atoms were shown with Grignard reagent. It showed also the interesting property of adding 1 mol of methyl alcohol when warmed with methyl alcohol containing mineral acid. The nature of these substances as well as their relationship to strophanthidin will be the subject for further studies.

EXPERIMENTAL.

Undephanthotriacid Dimethyl Ester.—A chilled solution of 10 gm. of anhydro- α -isostrophanthonic dimethyl ester in 1250 cc. of absolute acetone was treated with a cold solution of 18 gm. of KMnO_4 in 1500 cc. of the same solvent. The mixture was allowed to stand at 11° until all of the permanganate had been used up—which required about $5\frac{1}{2}$ hours. The precipitated manganese dioxide was collected as rapidly as possible on a large Buchner funnel and washed twice with absolute acetone, sucked down well, and quickly transferred to a bottle containing 1500 gm. of crushed ice. The mixture was shaken until all ice had melted. After filtration the manganese dioxide was again shaken up with 700 cc. of water and, after filtration, this operation was repeated with 500 cc. of water. The combined aqueous extracts were treated with 7 gm. of ammonium sulfate to neutralize any fixed alkali and concentrated under diminished pressure to about 400 cc. Any separated material was redissolved by careful addition of ammonia. On being acidified with acetic acid an amorphous precipitate formed, which crystallized in greater part on standing. This was collected with water. Since purification, as the acid, was difficult, the substance was usually converted as the crude acid into the ester which had excellent properties. For analysis the acid was repeatedly recrystallized from methyl alcohol from which it separated as slender, curved needles which were collected with cold, 50 per cent methyl alcohol. The substance melts at $179\text{--}180^\circ$ with slight preliminary sintering and gives the Legal test. It is soluble in the alcohols, chloroform, and acetone and more sparingly soluble in ether.

3.592 mg. substance: 2.265 mg. H_2O , 8.151 mg. CO_2 .

$\text{C}_{24}\text{H}_{32}\text{O}_9$. Calculated. C 62.03, H 6.95.

Found. " 61.88, " 7.05.

0.0422 gm. of substance was covered with 2 cc. of alcohol and titrated directly against phenolphthalein. Found, 0.911 cc. of 0.1 N NaOH. Calculated for 1 equivalent, 0.909 cc. 10 cc. of 0.1 N NaOH were then added and the solution was boiled for 4 hours and titrated back. 2.938 cc. of 0.1 N NaOH were consumed. Calculated for 3 equivalents (two ester and one lactone groups), 2.726 cc. The slight excess of that observed over the amount required by theory was due to slight simultaneous ring cleavage.

The resistance to ring cleavage on boiling with 0.1 N NaOH was shown in a series of saponification experiments. After 3 hours boiling 4.06 equivalents of base were consumed; after 5 hours boiling, 4.22 equivalents; and after 7 hours boiling, 4.36 equivalents were consumed. Complete cleavage could be accomplished by the use of N alkali as shown in saponification experiments with the neutral trimethyl ester.

Undephanthontriacid Trimethyl Ester.—The previous acid as directly obtained was treated with diazomethane in acetone solution. After evaporation of the solvent, the residue readily crystallized. Recrystallized from methyl alcohol the neutral ester forms long narrow platelets which melt at 154.5–155.5° and contain no solvent. It is easily soluble in acetone and chloroform and rather sparingly soluble in the cold alcohols and ether. It gives the Legal test but no coloration with ferric chloride. The ester reacted with both hydroxylamine and phenylhydrazine. All attempts to acylate it resulted in recovery of unchanged material, although the substance showed 1.6 active hydrogen atoms. Likewise, hydrogenation experiments proved negative. From 10 gm. of anhydro- α -isostrophanthonic dimethyl ester the yield of the ester was 2.7 to 3 gm.

3.241 mg. substance: 2.051 mg. H_2O , 7.460 mg. CO_2 .

3.318 " " : 2.175 " " 7.618 " "

3.348 " " : 3.443 " AgI.

3.068 " " : 4.490 " "

$C_{25}H_{34}O_8$. Calculated. C 62.73, H 7.17, OCH_3 19.45.

Found. " 62.77, " 7.10.

" " 62.61, " 7.33.

"

OCH_3 19.36.

"

" 19.30.

0.0496 gm. substance gave 4.3 cc. CH_4 (25°, 758 mm.) or 1.65 mols for mol. wt. 478.

0.0518 gm. substance gave 4.4 cc. CH_4 (25°, 758 mm.) or 1.62 mols.

Ring cleavage of the β -ketonic group was shown by the following titration experiments with N alkali.

0.0998 gm. substance was refluxed for 2 hours with 3 cc. of alcohol and 3 cc. of N NaOH and titrated back against phenolphthalein. Found, 0.98 cc. N NaOH. Calculated for 4 equivalents, 0.84 cc.; for 5 equivalents, 1.04 cc.

0.0892 gm. similarly refluxed for $2\frac{3}{4}$ hours consumed 0.918 cc. of N NaOH. Calculated for 5 equivalents, 0.933 cc.

Attempts to isolate the tetrabasic lactone acid resulting from the ring cleavage or acid decomposition of this β -keto ester were unsuccessful. Likewise attempts to prepare its ester resulted in non-crystalline substances.

Undephanthotriacid Trimethyl Ester Oxime.—The trimethyl ester when heated in methyl alcoholic solution with hydroxylamine hydrochloride and potassium acetate formed slowly the oxime. For complete reaction heating for 30 hours at 100° was necessary. The oxime separated slowly after dilution. Recrystallization from methyl alcohol gave six-sided pillars which melted at 226 – 227° and contained no solvent of crystallization. The substance was dimorphous since on occasion it separated as rectangular plates which melted at 188 – 189° and was also solvent-free. These forms were interconvertible.

4.299 mg. substance: 2.765 mg. H_2O , 9.603 mg. CO_2 .

5.380 " " : 0.5396 " N (Van Slyke).

5.486 " " : 0.5780 " " "

$C_{25}H_{35}O_5N$. Calculated. C 60.81, H 7.15, N 2.84.

Found. " 60.91, " 7.20.

" " N 2.86.

" " 2.87.

Undephanthotriacid Trimethyl Ester Phenylhydrazone.—0.2 gm. of the ester and 0.05 gm. of phenylhydrazine were warmed a few moments in 3 cc. of acetic acid. After removal of the acetic acid in a desiccator the residue was taken up in a few cc. of methyl alcohol. On careful dilution a colored amorphous precipitate formed, which was collected with water. The substance when recrystallized from methyl alcohol formed needles which melted at 196.5 – 197.5° and contained no solvent.

For analysis the substance was dried at 100° and 15 mm.

4.150 mg. substance: 2.510 mg. H₂O, 9.923 mg. CO₂.

3.070 " " : 1.914 " " 7.350 " "

C₃₁H₄₀O₈N₂. Calculated. C 65.46, H 7.10.

Found. " 65.20, " 6.77.

" " 65.29, " 6.98.

Undephanthontriacid Trimethyl Ester Phenyl-γ-Lactazam.—The phenylhydrazone was readily converted into the lactazam with the loss of methyl alcohol by the method of Michael.⁵ 0.145 gm. of the phenylhydrazone was suspended in 10 cc. of acetic acid and slightly warmed. To this was added 1 drop of concentrated hydrochloric acid. The deep orange color of the solution immediately became lighter and the suspended phenylhydrazone dissolved completely. After the mixture was heated on the steam bath for 15 minutes the acetic and hydrochloric acids were largely removed by evaporation in a desiccator. The oily residue was neutralized with dilute ammonia, giving an amorphous powder which was collected and recrystallized from dilute methyl alcohol. The substance was finally recrystallized from a small volume of methyl alcohol by strongly chilling. The lactazam separated as colorless, hairlike needles which contained no solvent and melted at 155–157°, then solidified, and remelted at 240–242°.

3.252 mg. substance: 1.918 mg. H₂O, 7.983 mg. CO₂.

2.758 " " : 1.626 " " 6.767 " "

C₃₀H₃₆O₇N₂. Calculated. C 67.13, H 6.77.

Found. " 66.94, " 6.60.

" " 66.83, " 6.59.

Undephanthontriacid Monomethyl Ester.—This ester was prepared in two ways: by partial saponification of the dimethyl ester and by oxidation of anhydro-α-isostrophanthonic monomethyl ester.

A solution of the dimethyl ester in methyl alcohol, after careful dilution with water, was made distinctly alkaline to phenolphthalein with dilute sodium hydroxide solution. After standing 1 hour at room temperature the solution was acidified to Congo red with hydrochloric acid, causing rapid crystallization. After

⁵ Michael, A., *Am. Chem. J.*, 1892, xiv, 519.

collection with 50 per cent methyl alcohol, the substance was recrystallized from methyl alcohol. It separated as rhombic leaflets which melted at 237–238°. For analysis the substance was dried at 100° and 15 mm.

0.0999 gm. substance: 0.0596 gm. H₂O, 0.2260 gm. CO₂.

C₂₃H₃₀O₈. Calculated. C 61.31, H 6.72.

Found. " 61.70, " 6.68.

The monomethyl ester was also obtained as follows: A suspension of 2 gm. of anhydro- α -isostrophanthonic monomethyl ester in 100 cc. of water was cleared by the addition of a few drops of ammonia. The solution was cooled to 0°, treated with 33 cc. of a 5.5 per cent potassium permanganate solution, and allowed to stand at 0° for 2½ hours when all the reagent had disappeared. After being warmed gently to coagulate the MnO₂, the mixture was filtered. The filtrate was carefully neutralized with acid, then concentrated to about 20 cc. Acidification to Congo red caused the separation of a resin followed by crystalline material. After standing 24 hours at room temperature, the substance was collected with water. Recrystallization from methyl alcohol gave 0.37 gm. of angular leaflets which were collected with cold 50 per cent MeOH. The substance melted at 233–235° which an additional recrystallization raised to 239–240°. In all properties this substance proved to be identical with that previously described and yielded the neutral trimethyl ester. For analysis the substance was dried at 100° and 15 mm.

5.406 mg. substance: 3.271 mg. H₂O, 12.154 mg. CO₂.

3.254 " " : 1.884 " " 7.302 " "

C₂₃H₃₀O₈. Calculated. C 61.31, H 6.72.

Found. " 61.28, " 6.77.

" " 61.20, " 6.48.

0.0976 gm. substance was covered with 5 cc. of alcohol and titrated directly against phenolphthalein with N NaOH. Calculated for 2 equivalents, 0.43 cc. Found, 0.43 cc. 5 cc. of N NaOH were then added and the solution was refluxed for 1½ hours and then titrated back. Calculated for 3 equivalents, 0.65 cc. Found, 0.62 cc. The consumption of 3 equivalents was caused by the saponification of one ester group, one lactone group, and ring cleavage.

Although in the case of the acid decomposition of the above

β -ketonic esters no crystalline reaction product was isolated, better results were obtained with the ketonic decomposition. Ketonic decomposition could not be accomplished by boiling the neutral ester of undephanthontriacid with dilute alcoholic sulfuric acid. Partial isomerization of the substance to a more soluble form occurred, which crystallized from concentrated methyl alcoholic solution as rosettes of leaflets which melted at 131–132°. The substance still gave the Legal reaction. No attempt was made to determine whether isomerization was complete and whether the substance isolated was homogeneous.

4.273 mg. substance: 2.760 mg. H_2O , 9.839 mg. CO_2 .

$C_{25}H_{34}O_9$. Calculated. C 62.72, H 7.17.

Found. " 62.79, " 7.22.

Duodephanthondiacid.—The ketonic decomposition could be accomplished readily, however, after saponification of the ester groups under the conditions previously described for the saponification and titration of the lactone and ester groups, but avoiding simultaneous ring cleavage; *i.e.*, by refluxing the ester with an excess of 0.1 N sodium hydroxide and alcohol for 3 hours. Titration showed that under these conditions only 4 equivalents of alkali had been consumed. The solution was then acidified to Congo red with hydrochloric acid and allowed to stand in a warm place. The ketone separated as masses of stout, wedge-shaped platelets which were easily soluble in dilute ammonia and melted at 260–262°. The substance was recrystallized from acetone from which it crystallized as masses of stout needles. The latter melted at 266–268°. From methyl alcohol it was obtained as stout rhombs or wedges and melted at 253–254°. The yield was 25 to 40 per cent of the starting material. The substance is rather difficultly soluble in chloroform and absolute acetone, somewhat more soluble in 90 per cent acetone, and fairly soluble in methyl and ethyl alcohols, especially when warmed. It gives no nitroprusside reaction.

For analysis it was dried at 100° and 15 mm.

3.447 mg. substance: 2.159 mg. H_2O , 8.105 mg. CO_2 .

3.634 " " : 2.370 " " 8.542 " "

$C_{21}H_{28}O_7$. Calculated. C 64.25, H 7.20.

Found. " 64.12, " 7.01.

" " 64.10, " 7.28.

That this is a dibasic lactone acid was shown by the titrations. 15.073 mg. of substance were covered with 1 cc. of alcohol and titrated directly against phenolphthalein with 0.1 N NaOH. Calculated for 2 equivalents, 0.768 cc. Found, 0.765 cc. 13.069 mg. of substance were directly titrated. Calculated for 2 equivalents, 0.666 cc. Found, 0.665 cc. 3 cc. of 0.1 N NaOH were then added and after refluxing for 3 hours the solution was titrated back. Calculated for 1 equivalent, 0.333. Found, 0.334 cc.

Duodephanthondiacid Dimethyl Ester.—The ester was obtained from the acid with diazomethane in acetone solution. Recrystallized from dilute acetone it separates as delicate needles melting at 166–167°. The ester is easily soluble in the alcohols, ether, acetone, and chloroform. It is insoluble in petroleic ether. For analysis it was dried at 100° and 15 mm.

3.680 mg. substance:	2.559 mg. H ₂ O,	8.881 mg. CO ₂ .
3.535 " " "	2.465 " " "	8.520 " " "
	C ₂₃ H ₃₂ O ₇ .	Calculated. C 65.67, H 7.68.
		Found. " 65.81, " 7.78.
		" 65.72, " 7.80.

Duodephanthondiacid Trimethyl Ester Oxime.—This was prepared in the usual manner from the keto ester in methyl alcoholic solution with hydroxylamine hydrochloride and sodium acetate. The oxime formed from methyl alcohol stout needles which melted at 187–189° and contained solvent of crystallization. For analysis it was dried at 100° and 15 mm.

3.034 mg. substance:	2.067 mg. H ₂ O,	7.033 mg. CO ₂ .
6.246 " " "	0.195 cc. N (26°, 750 mm.).	
	C ₂₃ H ₃₂ O ₇ N.	Calculated. C 63.40, H 7.64, N 3.22.
		Found. " 63.21, " 7.62, " 3.51.

The Lactonetriacid Dimethyl Ester, C₂₅H₃₂O₁₀.—When the aqueous filtrate from the precipitation with acetic acid of crude undephanthontriacid dimethyl ester from the reaction mixture was acidified to Congo red with hydrochloric acid, there occurred an immediate separation of amorphous material which became friable and largely crystalline on standing. The material was collected and washed with water. Its solubilities and properties were such that purification as the acid was difficult and it was found preferable to convert it into the neutral ester which had excellent properties. After repeated recrystallization from methyl alcohol,

the acid formed thin leaflets which melted at 188–190°. The substance contained water of crystallization, the last traces of which were very tenaciously held when it was attempted to dry the substance for analysis. For analysis it was dried at 100° and 15 mm.

4.425 mg. substance: 2.790 mg. H_2O , 9.800 mg. CO_2 .

$C_{28}H_{34}O_{10}$. Calculated. C 60.92, H 6.55.

Found. " 60.39, " 7.05.

18.298 mg. of dried substance were covered with 1 cc. of alcohol and titrated against phenolphthalein with 0.1 N NaOH. Calculated for 1 equivalent, 0.365 cc. Found, 0.386 cc.

3.5 cc. of 0.1 N NaOH were then added to the solution which was refluxed for 5 hours and titrated back. Calculated for 3 equivalents, 1.095 cc. Found, 1.139 cc.

The Lactonetriacid Trimethyl Ester, $C_{28}H_{34}O_{10}$.—The neutral ester was prepared from the previous crude substance with diazo methane in acetone solution. The yield from 10 gm. of anhydro- α -isostrophanthonic dimethyl ester was 0.5 to 0.7 gm. For recrystallization a solution in chloroform was diluted with methyl alcohol and then concentrated to remove the chloroform. The substance formed leaflets which melted at 236–237°. It was very sparingly soluble in the alcohols and acetone. The substance, contrary to the first oxidation product, did not give a Legal reaction. Attempts to form an oxime or phenylhydrazone were unsuccessful. Likewise, no acyl derivative could be obtained although it showed 1.89 active hydrogen atoms. No unsaturation could be detected by catalytic hydrogenation. However, as described below the elements of methyl alcohol may be added. Titration experiments confirmed also the presence of one lactone and three ester groups. But no ring cleavage due to a β -ketonic acid decomposition could be obtained with strong alkali.

3.910 mg. substance: 2.450 mg. H_2O , 8.855 mg. CO_2 .

4.485 " " : 2.757 " " 10.133 " "

3.250 " " : 4.595 " AgI.

$C_{28}H_{34}O_{10}$. Calculated. C 61.62, H 6.77, OCH_3 18.38.

Found. " 61.75, " 7.01.

" " 61.62, " 6.88.

"

OCH_3 18.67.

0.0519 gm. substance: 4.80 cc. CH_4 (23.5°, 762 mm.) or 1.89 mols for mol. wt. 506.

18.344 mg. of substance were refluxed with 1 cc. of alcohol and 4 cc. of 0.1 N NaOH for 3 hours and titrated against phenolphthalein. Calculated for 4 equivalents, 1.450 cc. Found, 1.448 cc.

0.1116 gm. of substance when boiled for 4 hours with 1 cc. of alcohol and 4 cc. of N NaOH consumed 0.898 cc. Calculated for 4 equivalents, 0.881 cc.

The Lactonetriacid, $C_{23}H_{28}O_{10}$.—When the above saponification mixtures were acidified to Congo red with hydrochloric acid, the tribasic lactoneacid slowly separated as stout needles. Recrystallized from dilute acetone, it formed long, flat plates which melted at 187–189°.

3.616 mg. substance:	1.967 mg. H_2O ,	7.885 mg. CO_2 .
3.074 " "	: 1.695 " "	6.731 " "
$C_{23}H_{28}O_{10}$.	Calculated.	C 59.46, H 6.08.
	Found.	" 59.46, " 6.08.
	"	" 59.71, " 6.16.

The above neutral trimethyl ester was dissolved in a small volume of chloroform and the solution was diluted with 10 volumes of dry methyl alcohol. A drop of hydrochloric acid (1.19) was added and the solution was concentrated to small volume on the water bath. After the solution had stood, stout needles gradually separated, which were collected with methyl alcohol. The substance was recrystallized by dissolving in chloroform, diluting with methyl alcohol, and then concentrating to remove the former. It melted at 193–195°. In solubilities, it resembled the parent ester. Analysis showed that addition of CH_3OH to the molecule had occurred, possibly on a masked carbonyl group.

3.041 mg. substance:	1.934 mg. H_2O ,	6.704 mg. CO_2 .
4.030 " "	: 2.600 " "	8.877 " "
3.454 " "	: 6.040 " "	AgI.
3.862 " "	: 6.792 " "	"
$C_{27}H_{38}O_{11}$.	Calculated.	C 60.19, H 7.12, OCH_3 23.05.
	Found.	" 60.12, " 7.12.
	"	" 60.07, " 7.21.
	"	OCH_3 23.08.
	"	" 23.21.

19.983 mg. of substance when refluxed for 4 hours with 1 cc. of alcohol and 3 cc. of 0.1 N NaOH consumed 1.463 cc. Calculated for 4 equivalents, 1.485.

When heated in a sealed tube at 100° for 1 hour with 50 per cent acetone containing 1 per cent of sulfuric acid, the methyl alcohol was removed and the parent substance, the lactonetriacid trimethyl ester, $C_{26}H_{34}O_{10}$, was recovered.

THE DIGITALIS GLUCOSIDES.

II. GITOXIGENIN AND ISOGITOXIGENIN.

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On a number of occasions¹ it has been pointed out that gitoxigenin² (anhydrodigitaligenin of Kraft³) gives a typical nitroprusside reaction which is no longer shown by its dihydro derivative, a fact which seemed to place it in the group of $\Delta^{\beta,\gamma}$ -lactones. We have attempted to demonstrate the correctness of this view by more direct means simultaneously with the series of investigations which has permitted us to obtain direct confirmation in the case of its closely related companion digitoxigenin⁴ and also of periplogenin.⁵ As recently shown, this has been accomplished with the latter substances by the method first employed in the case of strophanthidin;⁶ *i.e.*, by conversion into iso compounds which have then been shown to be lactones of the lactol forms of hydroxyaldehydes. In the case of gitoxigenin, however, a complication has persisted from the beginning of our attempts which must have its cause in a definite structural dissimilarity.

It has been pointed out already⁷ that gitoxigenin behaves differently on gentle treatment with 0.1 N alkali in the cold than digitoxigenin. The latter substance may be completely saponified, to the acid which may then be relactonized to digitoxigenin. In

¹ Jacobs, W. A., and Hoffmann, A., *J. Biol. Chem.*, 1926, lxxvii, 335.
Jacobs, W. A., Hoffmann, A., and Gustus, E. L., 1926, lxx, 1.

² Windaus, A., and Schwarte, G., *Ber. chem. Ges.*, 1925, lviii, 1517.

³ Kraft, F., *Arch. Pharm.*, 1912, ccl, 131.

⁴ Jacobs, W. A., and Gustus, E. L., *J. Biol. Chem.*, 1928, lxxviii, 573.

⁵ Jacobs, W. A., and Hoffmann, A., *J. Biol. Chem.*, 1928, lxxix, 519.

⁶ Jacobs, W. A., and Gustus, E. L., *J. Biol. Chem.*, 1927, lxxiv, 811.

⁷ Jacobs, W. A., Hoffmann, A., and Gustus, E. L., *J. Biol. Chem.*, 1926, lxx, 4.

the case of gitoxigenin, however, obscure secondary reactions seemed to accompany saponification with the formation of amorphous substances, and it was impossible to recover crystalline gitoxigenin on relactonization. More recently it has been found that this difficulty persists when the attempt is made to isomerize gitoxigenin with alcoholic alkali by the method which gives practically quantitative results in the case of digitoxigenin. Most of the substance was found to be changed to amorphous acid alteration products which could not be relactonized or crystallized. After a number of experiments, a modification of the method was devised which permitted the preparation of an isogitoxigenin, however, in only about 25 per cent yield. This substance like gitoxigenin was shown to possess the formula, $C_{23}H_{34}O_5$.

Isogitoxigenin in some respects resembles the other iso compounds. It no longer gives the Legal reaction and attempts to hydrogenate the substance were unsuccessful. Its formation was, therefore, unquestionably accompanied by a shift or disappearance of the double bond. With Grignard reagent only 3 active hydrogen atoms were found as against the 4 given by gitoxigenin itself. This is in agreement with the disappearance of the active hydrogen of the Δ^{β}, γ -lactone group but the isomerization apparently has not involved any of the three hydroxyl groups.

The similarity with other iso compounds, however, definitely ceased on further investigation. When saponified, isogitoxigenin yielded a crystalline *isogitoxigeninic acid* which could be readily relactonized. Contrary to the other iso acids such as isodigitoxigeninic acid it showed none of the reactions of a hydroxyaldehyde which may react either in the aldehydic or lactol form. As *isogitoxigeninic methyl ester* it did not form a semicarbazone and on oxidation with chromic acid yielded a neutral ester in which only one or possibly two secondary hydroxyl groups were oxidized to carbonyl with the formation of *isogitoxigenonic methyl ester*. The analytical figures which were obtained with this substance are in better agreement with those required by a diketone but this point will have to be confirmed. Saponification experiments with the substance showed the presence of only the ester group. No lactone group was formed as in the case of analogous experiments with isodigitoxigenin, isostrophanthidin, and isoperiplogenin.

Whereas the acids obtained by saponification of the normal iso

compounds were oxidized in the lactol form by hypobromite to give lactone acids, when isogitoxigeninic acid was oxidized with this reagent an anomalous oxidation product was obtained. Titration showed this substance to be a monobasic acid and no evidence of the formation of a lactone group could be obtained. Analyses of this acid were in best agreement with the formula $C_{21}H_{30}O_6$. This formula appears to have been confirmed by the analysis of its *methyl ester*, $C_{22}H_{32}O_6$. Since the acid showed no tendency to lactonize it would appear that degradation has involved that portion of the molecule belonging to the original lactone group of gitoxigenin. This, however, must await confirmation by further study.

With the facts available, it is difficult to assign a reason for the anomalous behavior of gitoxigenin and its iso compound. It appears probable that gitoxigenin is a $\Delta^{\beta,\gamma}$ -lactone like the other related aglucones but that the cause of the difference in its behavior towards alkali may be that unlike the others it has no hydroxyl group situated in a position γ or δ to the γ -carbon atom of the lactone group. By permitting the formation of a lactol derivative, such a hydroxyl may afford protection for an otherwise sensitive aldehyde group. Further data, however, are essential before much can be concluded in this regard.

Our analyses of gitoxigenin and its derivatives have been in best agreement with the formula $C_{23}H_{34}O_5$.⁸ This substance is, therefore, isomeric with periplogenin. Like periplogenin, strophanthidin, and digitoxigenin, it is a C_{23} derivative and this fact increases the probability of the very close structural relationship of all of these substances.

In connection with our investigations with gitoxigenin, we have obtained results which have conflicted in some ways with previous observations, in particular with those of Cloetta.⁹ This worker, after a long series of careful studies, succeeded in isolating from the so called gitalin fraction of the digitalis glucosides two glucosides which melted respectively at 282° and 245° , and for which on the basis of numerous micro analyses the formulæ $C_{40}H_{64}O_{14}$ and

⁸ Professor Windaus has informed us privately that he has concluded that gitoxigenin is a C_{23} derivative and not a C_{24} derivative as originally presented (see foot-note 2). (Windaus, A., *Chem. Zentr.*, 1928, ii, 669; *Nachr. Ges. Wissensch. Goettingen, Math.-physik. Klasse*, 1927, 422.)

⁹ Cloetta, M., *Arch. exp. Path. u. Pharmacol.*, 1926, cxii, 261.

$C_{17}H_{28}O_6$ respectively were derived. The former, called bigitalinum crist., was described as a glucoside of an aglucone bigitaligenin, $C_{22}H_{34}O_5$, and the latter, gitalinum crist., of a simpler aglucone, gitaligenin, $C_{11}H_{18}O_3$, which melted respectively at 232° and 222° . The so called bigitalinum crist. of melting point 282 is identical with the still impure anhydrogitalin of Kraft³ and the gitoxin of Windaus and Schwarte.² For the historical survey relating to this substance we must refer to the communication of the latter workers. Our own investigations have confirmed the view of Windaus and Schwarte that this substance possesses but one lactone group and one double bond. Careful hydrogenation experiments made with larger amounts of substance in connection with other investigations have demonstrated that gitoxigenin on hydrogenation gives rise to only one dihydro derivative and no tetrahydro compound as stated by Cloetta. The fact that gitoxigenin is a singly unsaturated monolactone is incompatible with the view of Cloetta that it has its origin in a doubling up of a C_{11} aglucone, gitaligenin.

It is to be regretted that we have not available a saponification value on this substance believed by Cloetta to be a C_{11} compound which would have at once decided its molecular size. The low molecular weights obtained by him may very well have been caused by cleavage of water (water of crystallization?) from his aglucone during the determination, and so have given an exalted molecular depression. This is particularly suggested by the fact that in all attempts to prepare derivatives of the low melting aglucone only derivatives of gitoxigenin were obtained by Cloetta.

We have attempted to prepare the new glucoside of Cloetta from different sources but without success. Our efforts have yielded only digitoxin or the latter contaminated with gitoxin or other gitoxigenin glucosides.

Gitoxigenin is a trihydroxy tetracyclic hydroaromatic $\Delta^{\beta,\gamma}$ -lactone, $C_{23}H_{34}O_5$.

Although a portion of the material used in the present studies was prepared directly from digitalis leaves, we are particularly indebted to E. Merck and Company, Darmstadt, for generous quantities of the insoluble by-product obtained in the manufacture of digitoxin.

EXPERIMENTAL.

Gitoxigenin (Anhydrogitoxigenin).—3 gm. of gitoxin which melted at 282–285° were hydrolyzed by refluxing in 50 cc. of 50 per cent alcohol and 0.5 cc. of hydrochloric acid (1.19). After 30 to 40 minutes solution was complete. Careful dilution and cooling caused separation of the aglucone which after collection with water weighed 1.15 gm.

After recrystallization from methyl alcohol, gitoxigenin was obtained in characteristic lustrous leaflets which melted at 231–232°, although occasionally samples were obtained which were not completely melted till 235°. Aside from a report of our analysis, we have nothing to add to the previous descriptions of this substance. For correct figures it was necessary to dry carefully at 100° and 15 mm. Frequently samples were obtained which gave figures 0.5 per cent low in carbon. This raises the question whether the low carbon figures frequently reported of substances which must have been largely gitoxigenin may not have been caused by the difficulty of drying, especially in cases of questionable purity.

4.805 mg. substance: 3.795 mg. H₂O, 12.420 mg. CO₂.

C₂₃H₃₄O₈. Calculated. C 70.72, H 8.78.

Found. " 70.48, " 8.83.

In confirmation of the fact that gitoxigenin as a $\Delta^{\beta,\gamma}$ -lactone gives the Legal test, were the results of the Zerewitinoff active H determination which showed 1 active H atom in addition to the 3 which belong to the three hydroxyl groups.

0.0468 gm. substance gave 12.1 cc. CH₄ (21.5°, 757.5 mm.), or 4.08 mols for mol. wt. 390.

Gitoxigenon.—1 gm. of gitoxigenin was dissolved in 20 cc. of acetic acid by gentle warming. The solution was quickly cooled and treated at once with 0.4 cc. or an excess of chromic acid solution (400 gm. of water, 80 gm. of sulfuric acid, and 53 gm. of chromic acid) with vigorous shaking. The reaction was prompt and after several minutes the mixture was diluted and shaken out with chloroform. The chloroform extract was washed with water, dilute carbonate solution, and then dried and concentrated. The crystalline residue was dissolved in a few cc. of 95 per cent alcohol

and allowed to crystallize, forming long leaflets which were collected with 50 per cent alcohol. The major portion remained in the mother liquor from which additional fractions were recovered. The ketone melted at 206–207° and contained approximately 1 mol of water. It is easily soluble in alcohol, chloroform, and acetone and very sparingly soluble in ether.

Air-Dry Substance.—Dried at 100° and 15 mm.

$C_{23}H_{32}O_5 \cdot H_2O$. Calculated. H_2O 4.43. Found. H_2O 3.97.

Anhydrous Substance.

Sample A. 0.0965 gm. substance: 0.0691 gm. H_2O , 0.2530 gm. CO_2 .

“ B. 0.0895 “ “ : 0.0644 “ “ 0.2336 “ “

“ C. 3.410 mg. “ : 2.460 mg. “ 8.936 mg. “

“ “ 3.052 “ “ : 2.272 “ “ 8.020 “ “

$C_{23}H_{32}O_5$. Calculated. C 71.08, H 8.31.

$C_{23}H_{30}O_5$. “ “ 71.46, “ 7.83.

Sample A. Found. C 71.50, H 8.01.

“ B. “ “ 71.19, “ 8.05.

“ C. “ “ 71.46, “ 8.07.

“ “ “ “ 71.63, “ 8.33.

The reaction product obtained from this substance and hydroxylamine did not crystallize.

Dihydrogitoxigenin.—2 gm. of gitoxigenin which were repeatedly recrystallized from glass-distilled alcohol were hydrogenated in alcoholic solution with 0.5 gm. of an active platinum black. Absorption occurred at first at the rate of 8 cc. per hour for the first 3 hours and then gradually diminished. After 24 hours practically 1 mol of H_2 had been absorbed and the absorption after that was practically negligible. In a second experiment in which 13 gm. of gitoxigenin were used with 2 gm. of catalyst, the absorption stopped after 48 hours at approximately 1 mol of H_2 . The combined experiments were filtered from catalyst and on concentration under diminished pressure yielded a thick paste of needles which melted at 239° and no evidence of the “Tafeln” of Cloetta was obtained. This material, as well as the substance which was recovered by further concentration of the mother liquor, was subjected to an exhaustive fractional recrystallization. In the end from a total of 15 gm. of starting material about 12.5 gm. of needles were obtained of high melting point. On two occasions during the fractionation process, when crystallization of smaller fractions occurred very slowly from a somewhat diluted solvent,

the substance was obtained partly as stout prisms or tables which melted at 195–197° and again at 205–207°. But when such material was recrystallized only the high melting form was obtained. The tables probably represent a hydrate of the substance.

After repeated recrystallization the substance melted at 249–250° after slight preliminary softening. The substance does not give the Legal test and the analysis showed definitely that it is a dihydro derivative.

Preparation A.	2.715 mg. substance:	2.293 mg. H ₂ O,	7.007 mg. CO ₂ .
"	" 2.373 "	" : 1.950 "	" 6.132 " "
"	B. 2.160 "	" : 1.767 "	" 5.579 " "
"	" 3.099 "	" : 2.597 "	" 8.016 " "
C ₂₃ H ₃₆ O ₅ . Calculated.		C 70.36, H 9.25.	
Found. Sample A.		" 70.39, " 9.45.	
"		" 70.46, " 9.20	
"		B. " 70.43, " 9.15.	
"		" 70.53, " 9.37.	

Isogitoxigenin.—After a number of experiments the following conditions were found to be most satisfactory for the preparation of this substance.¹⁰ 5 gm. of gitoxigenin were dissolved at 0° in a mixture of 25 cc. of dry pyridine and a solution of 1.2 gm. of potassium hydroxide in 25 cc. of dry methyl alcohol. After 4 hours at this temperature, during which the Legal test had become faint, the solution was mixed with several volumes of chloroform in a separatory funnel. The mixture was then shaken out several times with water which dissolved most of the amorphous acid alteration products as salts. Finally the chloroform solution was extracted with an excess of dilute sulfuric acid in order to remove the pyridine. The chloroform was finally washed and dried. On concentration the iso derivative readily crystallized, a process which was aided later by the addition of ether. The mother liquor on further manipulation gradually yielded an additional amount. The total yield was 1.3 gm. The acid reaction products which were extracted as the salts could be recovered only as resinous

¹⁰ In all probability, the experiments reported by H. Kiliani (*Ber. chem. Ges.*, 1915, xlviii, 341) are observations on the isomerization of gitoxigenin. His experiments are in agreement with our own on the isomerizing effect of alkali, and the crystalline substance obtained by him but not analyzed was possibly isogitoxigenin contaminated with ash.

material and this did not yield to further study. When recrystallized from dry chloroform, isogitoxigenin forms stout needles which melt at 249–250°. When recrystallized from alcohol it forms a heavy crust of prisms and 4 sided plates which melt at 218° with effervescence and contain small amounts (less than 1 mol) of solvent which it holds very tenaciously on being dried for analysis. It is readily soluble in methyl and ethyl alcohols and acetone and is less readily soluble in chloroform. It is very sparingly soluble in ether. The substance does not give the Legal test. Attempts to show the retention of a double bond by hydrogenation experiments were unsuccessful. The optical rotation in pyridine solution is very slight. $[\alpha]_D = -1^\circ$ ($c = 1.000$ in pyridine).

For analysis the substance was dried at 100° and 20 mm.

Sample A. 2.745 mg. substance: 2.166 mg. H_2O , 7.138 mg. CO_2 .

" " 2.768 " " : 2.210 " " 7.140 " "

" B. 3.082 " " : 2.480 " " 7.951 " "

$C_{23}H_{34}O_5$. Calculated. C 70.72, H 8.78.

Found. Sample A. " 70.86, " 8.84.

" " " " 70.34, " 8.93.

" " B. " 70.35, " 9.00.

0.0578 gm. gave 10.1 cc. CH_4 (21.5°, 757.5 mm.) or 2.76 mols for mol. wt. 390.

Isogitoxigeninic Acid.—1.4 gm. of isogitoxigenin were dissolved in 60 cc. of hot alcohol and diluted with an equal volume of water followed by 5 cc. of 10 per cent sodium hydroxide solution. After about 5 minutes the solution was cooled and diluted. On gentle acidification with acetic acid the acid separated as microscopic rods which were quickly collected with water. If allowed to stand long under the influence of free acid, lactonization gradually occurred. This was rapid with mineral acid. When recrystallized from alcohol it formed needles which slowly melted and effervesced at 146–149°. When dissolved in water with a few drops of ammonia and reprecipitated with acetic acid, it formed needles which melted at 152–153°. After being dried in a desiccator it retained 1.5 mols of water of crystallization. When the attempt was made to dry the substance for analysis at 100° and 15 mm., partial lactonization occurred. The analysis of the desiccator-dried substance is therefore reported.

3.329 mg. substance: 2.720 mg. H_2O , 7.770 mg. CO_2 .

3.769 " " : 3.082 " " 8.773 " "

$C_{23}H_{34}O_6 \cdot 1\frac{1}{2} H_2O$. Calculated. C 63.40, H 9.03.

Found. " 63.63, " 9.14.

" " 63.46, " 9.14.

Isogitoxigeninic Methyl Ester.—The acid was esterified in acetone suspension with diazomethane. The ester separated from dilute methyl alcohol as dendritic masses of plates which melted at 145° . It is soluble in the alcohols, particularly on being warmed, and less readily soluble in descending order in acetone, chloroform, and ether. The ester did not react with semicarbazide.

3.725 mg. substance: 3.130 mg. H_2O , 9.340 mg. CO_2 .

$C_{24}H_{32}O_6$. Calculated. C 68.20, H 9.07.

Found. " 68.37, " 9.40.

Isogitoxigenonic Methyl Ester.—0.3 gm. of the above ester was dissolved in 15 cc. of acetic acid. On addition of 1.5 cc. of Kiliani chromic acid solution at ordinary temperature rapid oxidation occurred and the reagent was in definite excess. The diluted mixture was extracted with chloroform. The extract was in turn washed with water, dilute sodium carbonate solution, and water and then after being dried was concentrated. The residue slowly crystallized. When dissolved in a few cc. of methyl alcohol and carefully diluted, the substance slowly crystallized and was collected with dilute methyl alcohol. This was recrystallized by concentrating its methyl alcoholic solution to a few cc. At low temperature the solution slowly deposited the ester as small prisms and rods which melted at 170 – 172° .

From the analysis and titration only one or possibly two secondary alcoholic groups were oxidized to carbonyl.

4.330 mg. substance: 3.260 mg. H_2O , 10.900 mg. CO_2 .

$C_{24}H_{32}O_6$. Calculated. C 68.52, H 8.63.

$C_{24}H_{34}O_6$. " " 68.86, " 8.19.

Found. " 68.64, " 8.42.

10.432 mg. of substance were treated with 1 cc. of alcohol and 2.5 cc. of 0.1 N NaOH. The mixture was refluxed for 4 hours and titrated back against phenolphthalein. Calculated for 1 equivalent, 0.248 cc. Found, 0.232 cc.

The Acid, $C_{21}H_{30}O_6$.—0.4 gm. of isogitoxigeninic acid was dissolved

in 4 cc. of water and a few drops of dilute sodium carbonate solution. The mixture was treated with 4 cc. of a solution of bromine in N sodium hydroxide solution which contained 60 mg. per cc. After standing 1 hour the mixture was acidified to Congo red with dilute sulfuric acid. A copious, amorphous precipitate formed, which rapidly crystallized. After collection with water the acid was recrystallized by careful dilution of its alcoholic solution. The substance which dissolved completely in dilute ammonia formed bundles of radiating needles which melted at 252–253°. It is soluble in descending order in methyl and ethyl alcohols in the cold and in acetone, and is sparingly soluble in chloroform. It is practically insoluble in ether.

For analysis the substance was dried at 100° and 15 mm.

Sample A.	2.667 mg. substance:	1.881 mg. H ₂ O,	6.525 mg. CO ₂ .
" "	2.559 "	" : 1.838 "	" " 6.242 " "
" "	2.840 "	" : 1.993 "	" " 6.970 " "
" B.	3.594 "	" : 2.645 "	" " 8.757 " "
 C ₂₁ H ₃₀ O ₆ . Calculated.			
			C 66.62, H 8.00.
	Found.	Sample A.	" 66.67, " 7.90.
	"	"	" 66.52, " 8.03.
	"	"	" 66.85, " 7.85.
	"	B.	" 66.45, " 8.24.

11.174 mg. of substance were covered with 1 cc. of alcohol and titrated directly against phenolphthalein with 0.1 N NaOH. Calculated for 1 equivalent, 0.295 cc. Found, 0.303 cc.

When an excess of alkali was added and the mixture was refluxed for 6 hours further consumption of alkali was negligible.

Methyl Ester of the Acid, C₂₁H₃₀O₆.—The ester was prepared from the acid in acetone solution with diazomethane. It crystallized slowly from dilute methyl alcohol as a crust of rosettes of stout plates or tables which contained solvent of crystallization. The air-dry substance effervesced at 105° and again crystallized, finally melting on further heating at 180°.

Air-Dry Substance.—Dried at 100° and 15 mm.

3.546 mg. substance: 0.216 mg. loss.

C₂₂H₃₂O₆ · 1½ H₂O. Calculated. H₂O, 6.44. Found. H₂O, 6.25.

3.240 mg. substance: 2.305 mg. H₂O, 7.996 mg. CO₂.

3.540 " : 2.737 " " 8.732 " "

C₂₂H₃₂O₆. Calculated. C 67.30, H 8.22.

Found. " 67.30, " 7.96.

" " 67.26, " 8.62.

BLOOD REGENERATION IN SEVERE ANEMIA.

XII. POTENT INFLUENCE OF INORGANIC ASH OF APRICOTS, LIVER, KIDNEY, AND PINEAPPLE.*

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About 8 years ago Robschuit-Robbins and Whipple at the Hooper Foundation in the University of California Medical School observed that apricot feeding had a potent influence in experimental anemia in dogs. These observations were not published because the reaction was so unexpected as to suggest the possibility of error. A few years later these observations were repeated and extended to show that apricots and peaches (fresh, dried, or canned) were quite potent in severe anemia due to hemorrhage in dogs (3). The addition of 200 gm. of this cooked fruit to the daily standard diet may cause an average output of 40 to 45 gm. of hemoglobin per 2 week period over and above the control periods. At this time some extracts (watery and alcoholic) were prepared from dried apricots and tested.

All the evidence pointed to *salts* as perhaps responsible for at least a part of this unexpected reaction. Some crude combustion products containing much carbon were then prepared and found to be potent in this experimental anemia. Finally we proceeded to the more complete combustion of the dried apricots¹ as described below and this pure inorganic material was likewise found to be potent, suggesting that some of the metals are concerned in the

* This work has been aided by a National Live Stock and Meat Board Fellowship of the National Research Council. We are indebted to the Rochester Packing Company for a liberal supply of certain meat products.

¹ We are indebted to the Taylor Instrument Company for the use of a high temperature muffle furnace in completing the first ashing of apricots.

reaction. Logically the potent organ tissues were next tested by ashing and feeding in the experimental anemia with almost identical results (2). Our results are not wholly in accord with the observations of the Wisconsin investigators (4) but the divergent results are to be explained by differences in the anemia or in the experimental animal. They study a nutritional anemia in rats and rabbits while we use a simple anemia due to hemorrhage in otherwise normal dogs.

Our general anemia program was outlined in the first papers of this series (5) and many important points there discussed need not be reviewed here. We stressed the importance of a *sustained maximal stimulus to hemoglobin production* in these dogs whose circulating hemoglobin level is maintained at 40 to 50 per cent hemoglobin, or approximately one-third normal for the dog. The surplus hemoglobin removed by bleeding to maintain this base line level is measured accurately and represents the capacity of the dog to form hemoglobin and red cells on the given food intake over and above the unknown red cell maintenance factor. These dogs are raised in our kennels and accustomed to the environment and experimental technique so that they lead normal, active lives undisturbed by the experimental observations, unless some note is made to the contrary. Uniformity of environment is very important and includes heat, ventilation, exercise, and isolation to minimize cross infection.

It is important to remember that these standard dogs are in a condition of sustained anemia (40 to 50 per cent hemoglobin) month after month, year after year. Some of the dogs have been *continuously anemic* as indicated for 4 years and are reacting quantitatively to various specific diet intakes in the 4th year as they did in the 1st year. The experiments given in the tables are taken out of the dogs' experimental history which is continuous. The figures given represent the observations made at the *end of any given week* so that the hemoglobin figures noted for the 1st week of ash feeding represent the production of hemoglobin due to 7 days of ash and bread feeding. It is necessary to refer to this point because Riecker (1) fell into error in attempting a critical review of our published results. He assumed that our figures referred to the beginning of the experimental week although it would seem that a careful reading of the papers under discussion would have obviated this mistake.

The *reserve storage of hemoglobin factors* is another important point which bears on the same interpretation of results. It is surprising to observe how difficult it may be to exhaust this emergency reserve in the healthy dog. We have made it a rule to produce our experimental anemia slowly over a 2 or 3 week period and to maintain this anemia for 2 or 3 months on the standard bread diet to insure the exhaustion of this reserve. During this period as much as 100 to 175 gm. of hemoglobin may be removed by bleeding, representing a tremendous reserve which can scarcely be stored as finished red blood cells. This reserve is probably stored as intermediates which on demand can be combined to form the finished blood hemoglobin. After this period the dog will react with a small and constant hemoglobin output to the standard bread diet and will react uniformly to various diet factors as given in preceding papers. If one does not appreciate this emergency reserve, quite unusual reactions to diet factors of low potency will be recorded and wrongly attributed to the diet rather than to the reserve storage.

Methods.

The general methods used in these experiments have been described in detail in the first (5) and fifth (6) papers of this series. The *standard bread* used in all these experiments is described as to ingredients and preparation. This bread contains wheat flour, starch, bran, sugar, cod liver oil, canned tomatoes, canned salmon, yeast, and salt mixture. Bread (S) which contains a little salmon was used in all the experiments here recorded. This bread is a complete diet for an adult dog and will maintain a dog in health for long periods of time, if not indefinitely. This ration keeps the hemoglobin production at a very low level, the average being close to 1 to 3 gm. of hemoglobin per week period over and above the unknown maintenance factor. There are individual differences and some dogs are constantly high in hemoglobin production on the basal rations as well as other diets. The *hemoglobin index* in these papers is a figure obtained by dividing the hemoglobin per cent by the red cell hematocrit in per cent. We believe this figure gives information of value as to the saturation of the red cell stroma with hemoglobin.

As has been pointed out by the Wisconsin investigators (4), the complete ashing of liver presents considerable difficulty. After being dried it may be burned down to a carbonaceous mass without trouble but when an attempt is made to ash this black material in crucibles, even at very high temperatures, the top layers sinter down, protecting most of the mass from oxidation. Moreover, the ash itself is formed as a melt and tends to be absorbed by the container, particularly if the latter is of porous composition. Because of this latter difficulty Hart and his coworkers mixed a large amount (4 gm. per 100 gm. of dried liver) of calcium carbonate with the liver before ashing. Some trouble was experienced in feeding ash extracts due to the presence of so much calcium.

We have been able to prepare *pure* ashes of liver (and other substances) without the addition of any foreign substance by the following procedure: Fresh beef liver, pig kidney, or dried apricots are weighed and placed in a large fire brick container with a well glazed surface. Free flames from several blow-torches are played on this material until it is reduced to a black carbon mass. At this point a stream of oxygen² is directed into the hot material, burning it down to a white-gray or bluish brown, glassy ash. This is then ground in a mortar, passed through a 40 mesh sieve, weighed, and fed in doses equivalent to the original fresh material. The material used in these experiments contains 4 to 8 per cent of carbon but it is reasonably certain that all organic compounds are broken down.

The pineapple ash was not made in our laboratory but obtained from a canning factory in the Hawaiian Islands through the courtesy of Dr. Benjamin Slater of the Eastman Kodak Company. It is a fine gray ash obtained by combustion of the skins and residues of the ripe pineapple.

Methods of Ash Analysis.

The following methods of analysis are taken from Official and Tentative Methods of Analysis of the Association of Official Agricultural Chemists, 1925, with some necessary changes.

Organic Matter.—A weighed sample of the ash as fed is placed in a crucible and ignited until all the organic matter is burned.

² We are indebted to Dr. R. P. Kennedy of the Department of Pathology for suggesting the use of oxygen in this connection.

The residue is weighed. The difference corresponds to the organic matter.

Silica.—A weighed sample of the ash, freed from organic matter, is evaporated with dilute hydrochloric acid on a steam bath in a weighed platinum crucible and heated for 1 hour. The residue is treated with hydrochloric acid and filtered. The remaining residue is washed with hot water. The filtrate is evaporated and treated as above. The combined residues are ignited in a platinum crucible, weighed, and then treated with a sulfuric-hydrofluoric acid mixture, which is driven off, the residue being ignited and weighed. The difference in weight corresponds to the SiO_2 . The residue remaining is iron and aluminum and is added to the subsequent iron and aluminum fraction.

Copper.—The filtrate from the silica determination is adjusted to the proper pH and treated with hydrogen sulfide. The precipitate after being washed with hydrogen sulfide water is digested with yellow ammonium sulfide to dissolve the antimony. The remaining residue is heated with nitric and sulfuric acids until the solution is colorless. It is then cooled, diluted with water and treated with bromine water. The excess is removed and the solution treated with ammonium hydroxide. The excess is removed and the solution treated with acetic acid. Potassium iodide is added after cooling, and the solution is titrated with 0.01 N sodium thiosulfate.

Antimony.—The solution of antimony sulfide from above is neutralized with hydrochloric acid, filtered, and the precipitate, after solution in strong hydrochloric acid, is precipitated with hydrogen sulfide. The precipitate is oxidized with potassium chlorate, potassium iodide is added, and the solution is titrated with standard sodium thiosulfate.

Iron and Aluminum.—The filtrate from the hydrogen sulfide precipitation is concentrated to 150 cc. and treated with ammonium hydroxide until alkaline to methyl orange. The precipitate of iron and aluminum hydroxides is filtered, washed, redissolved, reprecipitated, washed, dried, ignited, and weighed as the oxides.

The residue from the silica determination and the above are fused with potassium hydrogen sulfate, cooled, evaporated on the steam bath with sulfuric acid, and heated until SO_2 fumes are

evolved. The mixture is dissolved in water, treated with hydrogen sulfide to remove any platinum, and filtered. The iron in solution is reduced with SO_2 in the presence of CO_2 after the excess hydrogen sulfide is removed, and titrated with standard potassium permanganate solution.

The iron is calculated to the oxide, the phosphates to P_2O_5 (see below). These two weights are subtracted from the iron and aluminum fusions and the difference represents Al_2O_3 .

Calcium.—The filtrate from the iron and aluminum determination is concentrated, treated with oxalic acid, heated to boiling, and neutralized with ammonium hydroxide to methyl red. The precipitate formed is washed with ammonium oxalate solution, redissolved, and reprecipitated. After being filtered, washed, and dried, it is ignited and weighed as CaO .

Magnesium.—The filtrate from the calcium determination is treated with sodium ammonium phosphate, made alkaline with ammonium hydroxide, and allowed to stand 12 hours. The precipitate formed is redissolved and reprecipitated, washed with dilute ammonium hydroxide, ignited, and weighed as magnesium pyrophosphate.

Phosphates.—A weighed sample of ash is dissolved in dilute hydrochloric acid, treated with nitric acid, and evaporated to dryness. The residue is taken up in water, made alkaline with ammonium hydroxide, and then acid with nitric acid. The phosphates are precipitated with ammonium molybdate reagent, filtered, washed with hot water, dissolved in an excess standard alkali, and back titrated with standard acid to phenolphthalein.

Sodium and Potassium.—These are present but no quantitative determinations were made.

Experimental Observations.

On the whole these reactions are quite uniform and indicate that in this type of anemia the inorganic ash of various food products contains salts of evident potency which influence favorably the regeneration of new red blood cells and hemoglobin in these standard anemic dogs. It is probable, if not certain, that in this method of ash production we lose some material and volatilize some

TABLE 121.
Apricot Ash.

Diet periods 1 wk. each. Food, gm. per day.	Food con- sumed.	Wt.	Plas- ma vol- ume.	Red blood cells.	Color index.	Hb index.	Red blood cell hema- tocrit.	Blood Hb level.	Hb re- moved, bled.
Dog 24-2, bull, male, adult.									
	per cent	kg.	cc.	mil- lion			per cent	per cent	gm.
Bread 400, salmon 75.	100	16.5	901	5,3	0.42	1.94	23.3	45	1.3
Apricot ash 2.6 (200)*.	100	16.3	894	5,9	0.46	2.08	27.8	58	15.6
" " 2.6 (200)*.	100	16.3	878	6,2	0.52	2.01	25.9	52	29.5
Bread 450.....	94	16.0	854	6,0	0.48	2.00	21.6	43	14.6
" 450.....	96	16.2	900	5,1	0.50	2.16	18.9	42	11.2
" 450.....	100	16.6	1003	3,9	0.54	2.10	19.9	42	1.3
Dog 21-67, bull, male, adult; splenectomy.									
Bread 250, salmon 100, Klim 25.....	88	11.9	644	3,9	0.60	2.10	20.6	43	1.3
Apricot ash 2.6 (200)†.	93	12.0	600	4,8	0.64	2.16	22.5	49	16.0
" " 2.6 (200)†.	98	12.0	590	4,9	0.63	2.27	26.2	59	20.1
Bread 225, salmon 100, Klim 25.....	100	12.1	620	3,7	0.73	2.14	18.8	40	13.6
Bread 225, salmon 100, Klim 25.....	96	12.4	662	3,6	0.69	2.26	22.2	50	1.3
Dog 24-25, bull, male, adult.									
Bread 450, salmon 75.	89	14.1	760	4,2	0.57	2.04	23.6	48	1.5
Apricot ash 10 (230)‡.	98	14.2	852	4,4	0.66	2.07	23.8	49	17.2
" " 10 (230)‡.	100	14.4	847	4,4	0.60	1.88	26.2	49	13.6
Bread 450, salmon 75..	100	14.4	848	4,3	0.46	2.00	19.9	40	1.2
" 450, " 75..	99	14.6	885	4,8	0.53	1.86	24.5	46	15.1

Apricot ash given in gm. per day; figures in parentheses indicate in gm., equivalent in commercial dried apricots.

Klim = dried commercial milk powder. Salmon = commercial canned western salmon.

$$\text{Hemoglobin index} = \frac{\text{hemoglobin per cent}}{\text{red cell hematocrit per cent}}$$

* Bread 400, salmon 75, daily diet.

† Bread 225, salmon 100, Klim 25, daily diet.

‡ Bread 375, salmon 75, daily diet.

elements which may be potent, yet there are abundant potent materials remaining.

Table 121 shows the potency of apricot ash of various types. These are representative experiments of a considerable series. The stimulus to hemoglobin production per 2 week period yields from 42 to 65 gm. of hemoglobin above the control periods. The average of the whole series is about 40 to 50 gm. of hemoglobin production per 2 week period above the control periods. There are no notable fluctuations in the hemoglobin and color indices and the blood volume is not disturbed. Splenectomy has been performed on Dog 21-67 but this animal reacts like a normal dog to all these diet experiments. In this dog the end week shows a hemoglobin level of 50 which is much above the initial level of 43 and indicates probably an excess of about 10 gm. of hemoglobin which should be added to the figures of hemoglobin production in this experiment. The ash fed in the third experiment, Table 121, contained a large amount of carbon which explains the high ash weight.

Table 122 shows high, low, and average figures for hemoglobin regeneration due to liver ash feeding during 2 week periods above control periods—38 to 65 gm. of hemoglobin. There is considerable variation in the ash weight as representing whole liver, some ash concentrates representing unusually large amounts of whole liver. One recalls that a favorable diet of liver will cause a large output of hemoglobin during the 2nd week of feeding and a continuation of much hemoglobin production during the first and even second control after period. This "carry over" is a uniform reaction on most favorable diets and indicates that the body *stores* the various materials in these foods suitable for hemoglobin production and gives them up only during a subsequent unfavorable diet (control) period. These ash feeding experiments show the same "carry over" into 2 or even 3 weeks of the after period.

In checking any given experiment as to the output of hemoglobin due to any diet factor we observe the initial hemoglobin control level and the end hemoglobin control level. These figures in the various tables usually show variations of only 2 to 3 per cent hemoglobin which represent the inherent limitations of these experiments—a factor of error probably in the neighborhood of 5 gm. of hemoglobin. Occasionally wider variations are observed

TABLE 122.

Liver Ash.

Diet periods 1 wk. each. Food, gm. per day.	Food con- sumed.	Wt.	Plas- ma vol- ume.	Red blood cells.	Color index.	Hb index.	Red blood cell hema- tocrit.	Blood Hb level.	Hb re- moved, bled.
Dog 24-49, bull, female, adult.									
	per cent	kg.	cc.	mil- lion			per cent	per cent	gm.
Bread 350, salmon 75..	96	15.7	1010	4,3	0.51	1.91	22.8	44	1.2
Liver ash 3.4 (600)*...	96	15.9	1022	6,0	0.42	2.11	23.1	50	12.5
“ “ 3.4 (600)*...	100	16.0	1085	4,5	0.53	2.01	23.9	48	1.5
Bread 375, salmon 75.	100	15.5	966	5,4	0.53	2.17	25.7	56	18.5
“ 375, “ 75.	100	15.7	934	6,0	0.52	2.00	24.8	50	16.7
“ 400, “ 75.	100	15.7	966	4,1	0.54	2.04	21.4	44	1.3
Dog 25-24, coach, male, adult.									
Bread 400, salmon 75, Klim 25.....	100	12.5	790	5,0	0.45	1.94	23.2	45	1.4
Liver ash 4(1400)†...	100	12.7	744	5,3	0.46	1.91	25.9	49	1.5
“ “ 4(1400)†...	100	12.5	754	6,4	0.50	1.94	22.8	44	29.5
Bread 400, salmon 75, Klim 25.....	100	12.6	823	4,7	0.48	1.94	22.8	44	1.2
Bread 400, salmon 75, Klim 25.....	100	12.9	790	4,8	0.53	2.03	22.9	46	11.5
Bread 400, salmon 75, Klim 25.....	100	12.9	816	4,2	0.52	2.00	22.1	44	1.3
Dog 24-59, bull, male, adult.									
Bread 500, salmon 75..	100	14.9	944	5,9	0.40	1.96	23.9	47	1.4
Liver ash 1.3 (600)†...	100	15.0	1000	6,8	0.43	1.98	27.6	55	19.4
“ “ 1.3 (600)†...	100	15.2	894	6,9	0.41	1.91	25.8	49	17.2
Bread 500, salmon 75..	100	15.0	984	6,6	0.41	1.97	23.8	47	17.1
“ 550, “ 75..	100	15.5	1047	6,0	0.39	1.90	24.8	47	1.4
“ 550, “ 75..	100	15.7	952	6,9	0.39	1.86	22.7	42	16.1
“ 550, “ 75..	100	15.4	1026	5,8	0.38	1.90	23.4	44	1.4

Liver ash given in gm. per day; figures in parentheses indicate in gm., equivalent fresh tissue.

* Bread 350, salmon 75, daily diet.

† Bread 400, salmon 75, Klim 25, daily diet.

‡ Bread 500, salmon 75, daily diet.

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TABLE 123.
Kidney Ash.

Diet periods 1 wk. each. Food, gm. per day.	Food con- sumed.	Wt.	Plas- ma vol- ume.	Red blood cells.	Color index.	Hb index.	Red blood cell hema- tocrit.	Blood Hb level.	Hb re- moved, bled.
Dog 23-1, bull, male, adult.									
	per cent	kg.	cc.	mil- lion			per cent	per cent	gm.
Bread 475.....	100	16.2	936	5,9	0.41	2.00	23.8	48	1.4
Kidney ash 5.3(500).*	100	17.0	892	6,2	0.44	2.01	25.7	52	16.3
" " 5.3(500).*	100	16.5	844	6,3	0.44	2.13	25.1	53	15.9
Bread 475.....	100	16.5	917	6,0	0.43	2.14	24.1	52	14.2
" 475.....	100	16.4	861	5,5	0.39	1.95	21.8	43	1.4
Dog 24-25, bull, male, adult.									
Bread 425, salmon 50..	92	14.5	817	4,6	0.50	1.90	24.2	46	1.2
Kidney ash 5 (650)†..	96	15.0	779	5,0	0.51	2.13	26.3	56	13.7
" " 5 (650)†..	100	15.0	845	4,2	0.54	2.07	21.9	45	13.4
Bread 425, salmon 75..	98	15.0	808	5,1	0.49	2.04	25.2	51	14.2
" 425 " 100..	92	15.0	867	5,8	0.45	2.10	23.2	49	12.8
" 425 " 100..	100	15.0	854	3,8	0.47	2.03	17.8	36	1.0
Dog 24-45, bull, female, adult.									
Bread 400, salmon 75..	100	21.2	1159	4,6	0.51	2.11	22.3	47	2.1
Kidney ash 4 (520)‡..	100	21.0	1077	5,5	0.55	2.14	25.0	54	29.4
" " 4 (520)‡..	100	20.8	1137	4,9	0.60	2.14	21.8	47	26.2
Bread 350, salmon 75..	100	20.8	1106	4,4	0.61	2.16	19.0	41	15.8
" 350, " 75..	100	20.8	1169	4,4	0.57	2.19	23.2	51	13.8
" 350, " 75..	100	20.3	1083	5,0	0.47	2.24	21.1	47	1.4

Kidney ash given in gm. per day; figures in parentheses indicate in gm., equivalent fresh tissue.

* Bread 450, salmon 50, daily diet.

† Bread 400, salmon 75, daily diet.

‡ Bread 350, salmon 75, daily diet.

and allowance is made on the basis of experience in any given dog,—for example, Dog 24-25, Table 123, shows a final hemoglobin level of 36 per cent hemoglobin which is 10 points below the

TABLE 124.
Pineapple Ash.

Diet periods 1 wk. each. Food, gm. per day.	Food con- sumed.	Wt.	Pla- ma vol- ume.	Red blood cells.	Color index.	Hb index.	Red blood cell hema- tocrit.	Blood Hb level.	Hb re- moved, bled.
Dog 24-22, coach, female, adult.									
	<i>per cent</i>	<i>kg.</i>	<i>cc.</i>	<i>mil- lion</i>			<i>per cent</i>	<i>per cent</i>	<i>gm.</i>
Bread 300, salmon 75, Klim 25.....	100	13.1	794	5,4	0.40	1.84	23.1	43	1.3
Bread 300, salmon 75, Klim 25.....	98	13.0	754	5,2	0.42	1.83	24.1	44	1.3
Pineapple ash 5*.....	97	13.5	768	5,9	0.43	1.97	23.9	46	12.0
" " 5*.....	94	13.7	772	5,2	0.44	1.89	24.4	46	1.4
Bread 300, Klim 25, Salmon 75.....	92	13.4	752	5,4	0.43	1.90	24.1	46	1.3
Dog 24-26, bull, male, adult.									
Bread 275, salmon 100, Klim 25.....	100	10.4	637	4,1	0.50	2.16	19.1	41	1.3
Pineapple ash 10†.....	96	10.6	614	4,6	0.59	2.27	24.9	57	14.8
" " 8†.....	100	10.3	603	4,8	0.55	2.26	21.2	48	15.7
Bread 225, salmon 100, Klim 25.....	100	10.4	647	4,0	0.59	2.26	20.7	47	1.4

Pineapple ash given in gm. per day; a commercial product.

Hemoglobin index = $\frac{\text{hemoglobin per cent}}{\text{red cell hematocrit per cent}}$

* Bread 300, salmon 75, Klim 25, daily diet.

† Bread 225, salmon 100, Klim 25, daily diet.

initial control level. We may say therefore that this represents the bleeding of about 10 to 12 gm. of hemoglobin too much during the second control after period and we should deduct 10 gm. of

hemoglobin from the total of 48 gm. of hemoglobin recorded for this dog, due to kidney ash feeding.

In the interpretation of these tables it should be kept in mind that these standard anemic dogs on long control diet periods of standard bread will show an average output of 1 to 3 gm. of hemoglobin per week. This is the control base line level and the differences noted during the other periods we feel must be due to the added diet factors. The "*maintenance factor*," which is an unknown, represents the destruction of hemoglobin and red cells due to general circulatory wear and tear. This factor under these experimental conditions is approximately a constant due to constant factors of temperature, exercise, and cage environment.

TABLE 125.
Ash Analysis in Gm.

	Liver ash.		Kidney ash.		Apricot ash.	
	1 gm. crude as fed.	1 kg. fresh liver.	1 gm. crude as fed.	1 kg. fresh kidney.	1 gm. crude as fed.	200 gm. crude dried apricots.
Organic.....	0.0825		0.0831		0.0871	
SiO ₂	0.0142	0.0834	0.0068	0.0543	0.0071	0.0185
Fe.....	0.0099	0.0585	0.0136	0.1089	0.0186	0.0484
Al.....	0.0129	0.0759	0.0178	0.1420	0.1082	0.2813
Cu.....	0.0247	0.1450	0.0280	0.1015	0.0556	0.1445
Sb.....	0.00068	0.0040	0.00038	0.0032	0.00032	0.0008
Ca.....	0.00516	0.0303	0.0133	0.1060	0.0539	0.1401
Mg.....	0.0084	0.0494	0.0194	0.1540	0.0539	0.1401

Table 123 shows that kidney ash is much like liver ash and these two ashes could not be differentiated by this physiological test. It is quite potent in its influence upon hemoglobin regeneration under these experimental conditions.

Pineapple ash (Table 124) is much less potent in the form here tested than are the other ashes. Whether this is due to differences of preparation we cannot say but this seems unlikely. Small doses of this ash (5 gm. per day) have very little effect but larger doses (10 gm. per day) may show more hemoglobin output. This last dosage is close to the maximum which can be mixed with the standard bread without rendering the mixture unpalatable to the dog. In any case this ash seems to be less than half as potent as apricot ash.

Table 124 also shows considerable contrast in the *hemoglobin index*, the first dog with low index and the second with a high index. Fluctuations in the hemoglobin index are of great interest and have some significance as to the saturation of the red cell stroma with hemoglobin. At times there seems to be a fluctuation of this index due indirectly to diet factors and such changes we believe are of importance. We hope to ascertain what factors control the stroma production and eventually to dissociate stroma and hemoglobin production as can readily be done in bile secretion with dissociation of bile pigment and bile salt production.

Table 125 gives the average values for various ash analyses done in this laboratory. The method is outlined above. It is of interest to note that copper is highest in the apricot ash but abundant in all ashes analyzed. Iron is also abundant but more so in the kidney. This iron content depends in considerable measure upon the blood content of the organ as examined and can be removed to a large extent by careful perfusion of the fresh organ. It is known that hemoglobin by mouth is not very potent for new hemoglobin production in these experiments (7).

We have tested a considerable variety of salts, based on this ash analysis and have made artificial salt mixtures which are disappointing in that these materials are largely inert. These studies will be continued but the copper and iron salts are promising and their influence on hemoglobin must be understood. In this type of anemia the iron salts are more potent than are the copper salts tested (8). The copper salt reactions are given in the accompanying paper.

CONCLUSIONS.

Apricot feeding is surprisingly potent in standard anemic dogs and causes a large output of hemoglobin above control levels. This may amount to 40 to 45 gm. of hemoglobin per 2 week period.

The inorganic ash of dried apricots retains most of the potency of the whole apricot and can exert the same favorable influence upon red cell and hemoglobin regeneration when incorporated into the diet of standard anemic dogs.

The inorganic ash of beef liver and pig kidney is likewise potent in this type of experimental anemia but this ash contains only about half the potent factors present in the whole cooked liver or kidney. The liver or kidney ash added to the diet may increase the hemoglobin output by 40 to 70 gm. per 2 week period.

The inorganic ash of pineapple (commercial product) is but slightly potent even in rather large doses.

Analyses of the ash samples of apricot, liver, and kidney are tabulated. The figures for copper and iron are of especial interest. This study of inorganic constituents is being continued.

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BLOOD REGENERATION IN SEVERE ANEMIA.

XIII. INFLUENCE OF CERTAIN COPPER SALTS UPON HEMOGLOBIN OUTPUT.

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We may refer to the tabulated ash analyses of apricots, liver, and kidney in the preceding paper to show that *copper* is represented in the inorganic residues in considerable amounts. We do not propose a review of the literature on copper salts in metabolism but refer to papers by Flinn and Inouye (1) and McHargue (5, 6). We may refer to an important paper by Hart, Steenbock, Waddell, and Elvehjem (2), reporting the significant effect of copper salts in a nutritional anemia of rats. Our results are somewhat different, probably because the anemia in the case of our dogs is due to continued bleeding. The influence of copper tartrate or sulfate added to the standard diet in our experiments is slight and much less than iron salts or the ash of apricots, liver, or kidney.

Experiments have been reported (8) to show that various extracts of whole liver are potent when tested in our anemic standard dogs. Active substances are found in watery extracts, in alcoholic extracts, and in the extracted liver residues. More of these extract data are soon to be published but we believe all this evidence points to a *group of substances* rather than one single substance as being responsible for the increased hemoglobin production in experimental anemia due to bleeding in dogs. It is obvious from the ash feeding experiments that the salts of metals are important—they belong in the group of potent substances—but we cannot yet say that the ash potency resides in one salt or a salt mixture. The evidence in our experiments seems to favor the belief that a balanced salt mixture is responsible for the favorable

ash reaction and increased hemoglobin production in this type of anemia. We have shown (7) that various iron salts are potent in this type of anemia, more so than are the copper salts, as tabulated below.

The experiments of Mallory, Parker, and Nye (4) are of interest in this connection. They have shown that copper acetate is toxic to certain animals in the dosage employed and may produce liver cirrhosis. Their experiments were not directed toward the hemoglobin problem but toward the large problem of liver cirrhosis. Their experiments were done on rabbits chiefly but in their study they included guinea pigs which are resistant to the toxic action of copper, and rats and monkeys which react apparently somewhat like rabbits. Mallory in a recent paper (3) refers to these experiments and states that sheep are quite susceptible to copper poisoning with resultant liver cirrhosis. There is evidence that these doses of copper may cause some toxic hemolysis but the blood studies are not given in detail.

At this time it would be well to keep in mind the experiments of Mallory and his coworkers which indicate that copper salts may be toxic. The doses used by them are large but they note that some animals are much more susceptible than others and we do not know whether humans are more sensitive to copper than are animals nor do we know whether an anemic patient may be more sensitive than a normal person. This point should be emphasized to indicate reasons for caution in the administration of copper salts in human anemia, as a toxic cirrhosis would be more of a liability to a patient than even a stubborn anemia which might yield to suitable diet therapy.

Experimental Observations.

The methods used in these experiments are reviewed in the preceding paper. It is obvious from Table 131 that *copper sulfate* in small doses added to the standard bread ration does not call out any large production of new red blood cells and hemoglobin. Various doses of copper sulfate are given in this series of experiments and the amount of copper in the potent ash samples was taken as an index of the dosage to be used. The various ashes contain about 25 mg. of copper per gm. of ash. The dosages of copper in mg. of the metal are given in the tables in parentheses.

TABLE 131.
Copper Sulfate.

Diet periods 1 wk. each. Food, gm. per day.	Food con- sumed.	Wt.	Plas- ma vol- ume.	Red blood cells.	Color index.	Hb index.	Red blood cell hema- tocrit.	Blood Hb level.	Hb re- moved, bled.
Dog 24-45, bull, female, adult.									
	per cent	kg.	cc.	mil- lion			per cent	per cent	gm.
Bread 350, salmon 75..	100	19.3	1119	4,7	0.43	2.09	20.0	42	1.4
Copper sulfate 252 (65)*.....	100	19.0	1067	5,0	0.46	2.16	21.4	46	1.3
Copper sulfate 252 (65)*.....	100	19.2	1082	5,3	0.47	2.16	22.9	49	1.6
Bread 350, salmon 75..	100	18.8	1074	5,3	0.48	2.29	22.6	52	11.6
“ 350, “ 75..	100	18.8	1105	5,6	0.44	2.16	22.5	49	12.4
“ 350, “ 75..	100	18.9	1126	4,7	0.47	2.24	19.7	44	1.3
Dog 25-23, bull, male, adult.									
Bread 250, salmon 100, Klim 25.....	100	13.3	760	5,2	0.42	1.92	22.7	44	1.0
Copper sulfate 19 (5).†	100	13.4	879	5,3	0.43	1.96	23.1	45	1.4
“ “ 19 (5).†	100	13.6	764	5,9	0.46	1.88	19.5	37	14.7
“ “ 19 (5).†	100	13.5	803	5,3	0.41	1.79	24.4	44	1.4
Bread 300, salmon 100, Klim 25.....	95	13.3	816	5,3	0.40	1.80	23.4	42	1.3
Bread 300, salmon 100, Klim 25.....	100	13.7	815	6,4	0.36	1.78	25.9	46	1.4
Bread 300, salmon 100, Klim 25.....	100	13.7	738	6,7	0.41	1.90	21.6	41	13.6
Bread 350, salmon 100, Klim 25.....	100	13.9	852	6,5	0.37	1.80	26.4	48	1.4
Copper sulfate 252 (65)†.....	86	13.5	758	6,4	0.39	2.01	24.8	50	1.7
Copper sulfate 252 (65)†.....	93	13.3	769	6,9	0.36	1.96	25.6	50	1.5
Bread 350, salmon 75, Klim 25.....	100	13.5	758	7,2	0.32	1.79	26.0	46	0
Bread 350, salmon 75, Klim 25.....	100	13.6	809	8,0	0.37	2.00	21.9	44	15.4

TABLE 131—*Concluded.*

Diet periods 1 wk. each. Food, gm. per day.	Food con- sumed.	Wt.	Plas- ma vol- ume.	Red blood cells.	Color index.	Hb index.	Red blood cell hema- tocrit.	Blood Hb level.	Hb re- moved, bled.
Dog 25-23, bull, male, adult—Continued.									
	per cent	kg.	cc.	mil- lion			per cent	per cent	gm.
Bread 400, salmon 75, Klim 25.....	100	14.0	886	7,0	0.37	2.08	21.5	45	12.8
Bread 400, salmon 75, Klim 25.....	91	13.9	814	6,6	0.38	2.01	20.8	42	12.4
Bread 400, salmon 75, Klim 25.....	100	14.0	821	6,0	0.34	1.94	21.4	41	1.2

Metal salts given in mg. per day; figures in parentheses indicate mg. of the metal.

Klim = dried commercial milk powder. Salmon = commercial canned western salmon.

$$\text{Hemoglobin index} = \frac{\text{hemoglobin per cent}}{\text{red cell hematocrit per cent}}.$$

* Bread 350, salmon 100, daily diet.

† Bread 275, Klim 25, salmon 100, daily diet.

‡ Bread 350, salmon 100, Klim 15.

The first experiment (Dog 24-45, Table 131) shows a slight increase in hemoglobin output in the 2 week after period, amounting to about 20 gm. of hemoglobin. This we may assume is due to 65 mg. of copper as metal or 252 mg. of copper sulfate as salt, given each day mixed with the standard bread. Doses of the copper sulfate larger than this usually cause lack of appetite and automatically diminish salt ingestion. The second experiment (Dog 25-23) shows the slight effect due to a small dose of copper sulfate—approximately 12 gm. of hemoglobin production above the control period. The second copper salt feeding of larger dosage yields a greater increase in hemoglobin production—about 30 gm. above control periods.

Copper tartrate is no more potent than copper sulfate and the two experiments in Table 132 show only 10 to 12 gm. of hemoglobin production above the control periods. The amounts given are 40 and 60 mg. of copper metal fed daily. Even the smaller dose caused some disinclination for food. There are no noteworthy changes in plasma volume although some salts cause a very

notable shrinkage of plasma volume in these standard anemic dogs. There are great fluctuations in the hemoglobin index which

TABLE 132.
Copper Tartrate.

Diet periods 1 wk. each. Food, gm. per day.	Food con- sumed.	Wt.	Plas- ma vol- ume.	Red blood cells.	Color index.	Hb index.	Red blood cell hema- tocrit.	Blood Hb level.	Hb re- moved, bled.
Dog 23-3, bull, female, adult.									
	per cent	kg.	cc.	mil- lion			per cent	per cent	gm.
Bread 325, salmon 100.	93	14.3	770	4,5	0.52	2.19	21.3	47	1.5
Copper tartrate 169 (40)*.....	77	14.0	746	4,6	0.52	2.16	21.9	47	1.5
Copper tartrate 169 (40)*.....	95	14.0	716	5,3	0.48	2.31	17.9	41	11.3
Bread 200, salmon 100, Klim 25.....	100	13.9	752	4,6	0.46	2.16	19.8	43	1.3
Bread 250, salmon 100, Klim 25.....	100	14.3	722	4,6	0.53	2.26	21.6	49	1.5
Dog 25-23, bull, male, adult.									
Bread 350, salmon 100, Klim 25.....	100	14.5	920	5,4	0.37	2.00	19.8	40	1.2
Copper tartrate 252 (60)†.....	100	14.3	850	6,5	0.49	2.11	22.0	46	14.9
Copper tartrate 252 (60)†.....	100	14.3	864	5,8	0.38	1.92	22.7	44	1.3
Bread 350, salmon 100, Klim 25.....	100	14.3	773	5,6	0.40	1.88	23.7	44	1.4

Metal salts given in mg. per day; figures in parentheses indicate mg. of the metal.

* Bread 175, salmon 100, Klim 25, daily diet.

† Bread 300, salmon 100, Klim 25, daily diet.

we are at a loss to explain. The influence of copper feeding at times is late in making its appearance. It is not as uniformly

prompt as liver feeding for example. This is true for iron feeding in some experiments—also for various salt mixture feeding experiments which have not yet been reported in detail.

Table 133 is of especial interest as it shows the combined effect of copper and iron salts added to the standard diet. We have accumulated a large amount of experimental data on iron feeding with a wide variety of dosage and salts. We may refer to some published experiments (7) and more data will soon be published. The question of iron salts and their reaction in the body in large doses, far above the needs for replacement, is a complex problem which calls for a large amount of experimental work. These iron salts seem to have some "salt action" or "catalytic effect" like certain salt mixtures which do not enter into the actual construction of the complex hemoglobin molecule.

At any rate it is obvious that copper salts combined with iron are much more potent than copper salts alone when added to standard diet in anemic dogs. There is a notable increase in hemoglobin output above the base line control level. This amounts to from 40 to 80 gm. per 2 week period in the experiments of Table 133. The first experiment (Dog 24-25) appears to show an output of only 28 gm. of hemoglobin as a result of 20 mg. of copper metal and 36 mg. of iron metal given daily, but we note that the initial level of 37 per cent hemoglobin is 9 points lower than the end level of 46 per cent hemoglobin. To allow for this we may assume about 10 gm. of hemoglobin produced to make up this difference or a total of $28 + 10$ gm. of total hemoglobin output due to the iron and copper feeding.

The second experiment (Dog 24-2, Table 133) shows an unusually favorable reaction to copper and iron feeding in fairly liberal dosage. The total increase in hemoglobin production due to 2 weeks mineral feeding in this dog reaches the high total of 80 gm. of hemoglobin. From time to time we note these unusual reactions and as yet can give no adequate explanation. This experiment is not given as typical but to illustrate an unusually favorable reaction. We have observed repeatedly by contrast certain dogs which at times give absolutely no reaction to iron or various salt mixture feeding. Eventually it may be discovered that the preceding diet periods, even 3 to 6 weeks before, may be in part responsible.

TABLE 133.
Iron Chloride and Copper Tartrate.

Diet periods 1 wk. each. Food, gm. per day.	Food con- sumed.	Wt.	Plas- ma vol- ume.	Red blood cells.	Color index.	Hb index.	Red blood cell hema- tocrit.	Blood Hb level.	Hb re- moved, bled.
Dog 24-25, bull, male, adult.									
	per cent	kg.	cc.	mil- lion			per cent	per cent	gm.
FeCl ₃ 176.5 (36), cop- per tartrate 84 (20).*	94	14.5	826	3,9	0.47	2.13	17.5	37	1.1
FeCl ₃ 176.5 (36), cop- per tartrate 84 (20).*	100	15.0	880	4,9	0.48	2.13	22.3	47	1.4
Bread 250, salmon 100, Klim 25.....	100	14.4	822	5,6	0.55	2.10	24.3	51	29.7
Bread 250, salmon 100, Klim 25.....	100	14.4	847	4,7	0.51	2.24	21.4	48	1.4
Bread 250, salmon 100, Klim 25.....	100	14.0	832	4,5	0.51	2.10	22.1	46	1.4
Dog 24-2, bull, male, adult.									
Bread 350, salmon 100, Klim 25.....	78	15.8	890	5,3	0.42	2.01	22.3	45	1.4
FeCl ₃ 353 (73), cop- per tartrate 164 (40)†.....	100	16.5	902	6,3	0.46	2.24	27.9	63	28.7
FeCl ₃ 353 (73), cop- per tartrate 164 (40)†.....	100	16.7	952	5,2	0.47	2.29	21.5	49	18.6
Bread 300, salmon 100, Klim 25.....	100	16.9	938	5,5	0.52	2.27	23.3	53	27.1
Bread 300, salmon 100, Klim 25.....	100	16.7	925	5,0	0.47	2.11	22.2	47	1.5
Bread 300, salmon 100, Klim 25.....	100	16.7	938	5,7	0.50	2.07	19.8	41	15.9
Bread 300, salmon 100, Klim 25.....	100	16.9	1008	4,4	0.50	2.27	19.2	44	1.3

TABLE 133—*Concluded.*

Diet periods 1 wk. each. Food, gm. per day.	Food con- sumed.	Wt.	Plas- ma vol- ume.	Red blood cells.	Color index.	Hb index.	Red blood cell hema- tocrit.	Blood Hb level.	Hb re- moved, bled.
Dog 24-26, bull, male, adult.									
	per cent	kg.	cc.	mil- lion			per cent	per cent	gm.
Bread 275, salmon 100, Klim 25.....	100	10.9	638	4,2	0.51	2.22	19.3	43	1.3
FeCl ₃ 705 (146), cop- per tartrate 164 (40)†.....	100	11.0	608	5,0	0.46	2.11	21.8	46	1.3
FeCl ₃ 705 (146), cop- per tartrate 164 (40)†.....	100	11.2	628	6,0	0.50	2.19	21.0	46	29.6
Bread 300, salmon 100, Klim 25.....	91	10.9	660	4,5	0.51	2.19	21.0	46	1.4
Bread 275, salmon 100, Klim 25.....	82	10.6	620	4,6	0.57	2.27	19.2	44	13.4
Bread 275, salmon 100, Klim 25.....	100	10.4	637	4,1	0.50	2.16	19.1	41	1.3

Metal salts given in mg. per day; figures in parentheses indicate mg. of the metal.

* Bread 250, salmon 100, Klim 25, daily diet.

† Bread 300, salmon 50, daily diet.

‡ Bread 300, salmon 100, Klim 25, daily diet.

The third experiment (Dog 24-26, Table 133) shows an average reaction due to a large dose of ferric chloride and an average dose of copper tartrate. It is fair to say that the increased output of hemoglobin above control periods (about 40 gm. in this experiment) is little if any above the expected reaction due to similar iron feeding alone.

The last experiment (Table 134) shows a combined effect of copper and zinc. The resultant increase in hemoglobin production above control level of 20 gm. is no more than we should expect from copper alone. Zinc alone may give us as favorable a reaction as the copper alone (9) but when combined the effect is minimal.

It should be kept in mind that the standard bread used in our

experiments contains iron in amounts probably adequate for the usual maintenance factor. As fed the bread contains 20 mg. of iron as metal per 300 gm. of whole bread.

TABLE 134.
Zinc Chloride and Copper Tartrate.

Diet periods 1 wk. each. Food, gm. per day.	Food con- sumed.	Wt.	Plas- ma vol- ume.	Red blood cells.	Color index.	Hb index.	Red blood cell hema- tocrit.	Blood Hb level.	Hb re- moved, bled.
Dog 23-1, bull, male, adult.									
	per cent	kg.	cc.	mil- lion			per cent	per cent	gm.
Bread 400, salmon 100.	100	17.4	925	5,4	0.42	2.24	19.9	45	1.4
Zinc chloride 51 (24), copper tartrate 169 (40)*.....	100	17.5	1058	5,4	0.40	2.16	20.2	44	1.3
Zinc chloride 51 (24), copper tartrate 169 (40)*.....	100	17.6	988	5,5	0.46	2.24	25.3	57	11.2
Bread 400, salmon 100.	100	17.5	972	5,6	0.43	2.01	24.1	49	1.5
“ 400, “ 100.	100	17.9	987	6,2	0.42	2.07	23.1	47	12.9
“ 400, “ 100.	100	17.8	1047	5,7	0.43	2.19	22.0	48	1.5

Metal salts given in mg. per day; figures in parentheses indicate mg. of the metal.

$$\text{Hemoglobin index} = \frac{\text{hemoglobin per cent}}{\text{red cell hematocrit per cent}}.$$

* Bread 400, salmon 100, daily diet.

CONCLUSIONS.

Certain metals and their salts when added to the standard diet of these anemic dogs have a distinct influence and increase hemoglobin production.

Copper has a positive effect, although moderate in degree, and usually increases somewhat the hemoglobin production above control levels.

The iron salt effect is much more notable in our experiments than the copper effect.

Combined copper and iron feeding may at times have an unusually favorable effect or again may not exceed the favorable influence of the iron salt feeding alone.

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DETERMINATION OF THE TYROSINE CONTENT OF PROTEINS.

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In 1925, I published a method for the quantitative colorimetric determination of tyrosine.¹ Up to that time tyrosine had been determined either by actual isolation or by colorimetric procedures that were applied to the crude hydrolysates. The colorimetric procedures employed were not *certainly* characteristic for tyrosine and reactions that are allowed to proceed in the presence of 95 or more per cent of compounds that are chemically similar to tyrosine are apt to be influenced by these other compounds.

The colorimetric procedure devised by K. K. Koessler and myself² is unusually characteristic for tyrosine, for, although all phenols and many other compounds give colors with diazotized sulfanilic acid in alkaline solution, tyrosine and tyramine are peculiar in that they give very little color, in dilute solution, until these solutions are rendered strongly alkaline and then treated with hydroxylamine. This color augmentation has, thus far, been observed only with tyrosine, tyramine, and with two apparently unrelated compounds, acetone and acetoacetic ester, both enols; so it is rather characteristic for tyrosine. This reaction could not, however, be employed on a crude protein hydrolysate because any amino compound, in sufficient concentration, will give a *yellow* color with alkaline diazotized sulfanilic acid; and that makes a reading of the purple color, due to tyrosine, practically impossible. I sought, therefore, for a method that would separate tyrosine from the bulk of other amino acids and found that when tyrosine is boiled in acetic acid solution with mercuric acetate and the cooled

¹ Hanke, M. T., *J. Biol. Chem.*, 1925, lxvi, 475.

² Hanke, M. T., and Koessler, K. K., *J. Biol. Chem.*, 1922, l, 235.

liquid treated with sodium chloride, a white precipitate is obtained that contains all but traces of the tyrosine.¹ Other amino acids are also precipitated to some extent, but they are *usually* present in such small concentrations that the colorimetric determination of tyrosine is not interfered with. In short, the mercury treatment is *not* a *specific* method for precipitating tyrosine. It is merely a method for eliminating the *bulk* of other amino acids so that our colorimetric procedure can be used for the estimation of tyrosine. There is never any doubt as to the efficiency of the mercury treatment in any given case because, if other amino acids have not been sufficiently removed, an intense yellow color develops during the 5 minute reaction period and, when this is the case, a determination will be of no value because the results will invariably be *high*. The values recorded in my earlier paper were all obtained on samples that gave ideal results. There was, in none of these cases, the slightest indication that the interfering substances were present. The color finally obtained was free from extraneous color and the values recorded most certainly represented tyrosine. Samples in which this ideal condition did not exist, and there were a few such, were discarded.

Joseph Looney, in 1926,² expressed the opinion that results obtained with my method are worthless. The mode of procedure is not criticized. Looney believes that *the colorimetric method* employed for the determination of tyrosine in the mercury precipitate *gives low results* because of two, what he considers to be facts; namely, (1) cystine and tryptophane are precipitated by mercuric acetate and will appear, together with tyrosine, in the mercury precipitate; (2) at least one of these amino acids, tryptophane, interferes seriously with the color production by tyrosine, the depth of the color being greatly *reduced*.

I readily concede that cystine and tryptophane are precipitated by mercuric acetate. Cystine is, however, just one of the amino acids that does *not* interfere with the color production by either histidine or tyrosine unless a large amount of cystine is present. Tryptophane is not present, in appreciable amounts, in acid protein hydrolysates (see "Experimental"). Looney was, evidently, not thoroughly familiar with our colorimetric process.

² Looney, J. M., *J. Biol. Chem.*, 1926, lxi, 519.

On page 530 of the article cited³ we read: "The color produced by 3 mg. of tyrosine alone is more than four times as deep as the color produced by 3 mg. of tyrosine plus 1 mg. of tryptophane." The method that I employed is very delicate, and the reagents are very dilute. It is not practical to conduct determinations with more than 0.03 mg. of tyrosine, and I usually employ only 0.02 mg. A very intense color is obtained with 0.03 mg. of tyrosine, almost too intense to read; and the reagent would surely be insufficient to give quantitative color production with 3 mg. Looney was using concentrations of tyrosine that more than exhausted the reactive components of the reagent, so that the colors obtained were far from quantitative. The addition of a second substance, tryptophane, that also reacts with the reagent, would, of course, *reduce* the color production by the tyrosine.

EXPERIMENTAL.⁴

The greater part of the work recorded here was performed on casein and crystallized egg albumin that were prepared in this laboratory. Three methods of hydrolysis were employed; namely, sulfuric acid, hydrochloric acid, and sodium hydroxide.

Hydrolysis with Sulfuric Acid.—Mix 5 gm. of vacuum-dried protein with 200 cc. of water and 30 cc. of 95 per cent sulfuric acid, and reflux for 30 to 40 hours over an electrically heated sand bath. Dilute to 5000 cc. and remove the sulfuric acid with barium hydroxide. Adjust so that the liquid contains just a *trace* of sulfuric acid. Filter, and wash the precipitate carefully with hot water. Concentrate in a glass dish on the water bath. Dilute to exactly 1000 cc. 200 cc. portions of this liquid, containing 1 gm. of hydrolyzed protein, are freed from water by evaporation in glass dishes. Preserve in a vacuum desiccator over calcium chloride.

Hydrolysis with Hydrochloric Acid.—Mix 5 gm. of vacuum-dried protein with 65 cc. of 37 per cent hydrochloric acid and 60 cc. of water and boil for 30 hours. The dark-colored solution is subjected to a distillation *in vacuo*. The last traces of free hydrochloric acid are removed by adding 5 cc. of water through the

⁴ All experiments have been conducted in duplicate unless otherwise specified. When only one set of figures is given, this represents the mean of two identical or nearly identical values.

ebullition tube and continuing the distillation. This is repeated three times. The dark brown residue is dissolved in 500 cc. of water. Add a suspension of carefully washed silver carbonate prepared from 10 gm. of silver nitrate. The clear supernatant liquid usually contains a trace of silver. Add 6 N hydrochloric acid, drop by drop, with agitation, until the supernatant liquid gives a test for chloride. Heat on the water bath and test again. The clear, pale colored, supernatant liquid should finally contain just a trace of chloride. Filter. Wash the precipitate carefully with hot water and dilute the filtrate to exactly 1000 cc. 200 cc. portions of this liquid, containing 1 gm. of hydrolyzed protein, are freed from water by evaporation in glass dishes. Preserve in a vacuum desiccator over calcium chloride.

Hydrolysis with Sodium Hydroxide.—The method described by Folin and Ciocalteu⁵ was followed exactly, excepting that I did not find the silver spirals to be essential and found a sand bath very satisfactory for boiling the alkaline solution. Hydrolyses were conducted on 5 gm. portions of protein.

Is Tryptophane Destroyed Completely by Acid Hydrolysis?

1 gm. portions of egg albumin and of casein that had been hydrolyzed by the hydrochloric acid and by the sulfuric acid process respectively, were treated with 10 cc. of N H_2SO_4 , transferred to volumetric flasks, and diluted to 100 cc. The vanillin method described by Kraus,⁶ which I have found to give excellent results with pure solutions of tryptophane, was used to determine the tryptophane content of these hydrolysates.

Of the hydrolysate, 1 cc. was mixed with 0.2 cc. of the 0.5 per cent vanillin solution and 7.5 cc. of 37 per cent hydrochloric acid. The liquid was practically colorless, at the end of 24 hours, in every case. Since 0.01 mg. of tryptophane still gives a decided color under these conditions, the 1 gm. portions of hydrolyzed protein must have contained less than 1 mg. of tryptophane.

A sulfuric or hydrochloric acid hydrolysis of either egg albumin or casein, conducted as described in this paper, leads to a complete destruction of the tryptophane.

⁵ Folin, O., and Ciocalteu, V., *J. Biol. Chem.*, 1927, lxxiii, 627.

⁶ Kraus, I., *J. Biol. Chem.*, 1925, lxiii, 157.

Does the Presence of Tryptophane Interfere with the Color Production by Tyrosine in the Process of Hanke and Koessler?

1. Tyrosine (1.0 mg.) and tryptophane (1.0 mg.) were mixed and diluted with water to 10 cc. Of this solution 0.1 cc. was used for the colorimetric tyrosine determinations² which indicated the presence of exactly 1.0 mg. in the entire liquid. This experiment was conducted in triplicate.

Accurate tyrosine values are obtained when the tyrosine is mixed with its own weight of tryptophane.

2. Tyrosine (1.0 mg.) and tryptophane (2.0 mg.) were mixed and diluted with water to 10 cc. Tyrosine was determined colorimetrically on 0.1 cc. portions of the liquid. The color obtained was distinctly *yellow* as compared with that obtained with pure tyrosine and the color intensity indicated the presence of 0.1125 mg. of tyrosine, or 112.5 per cent of the amount actually present.

3. A similar experiment was conducted on a mixture of 1.0 mg. of tyrosine and 3.0 mg. of tryptophane. The color obtained was so far off shade that an accurate comparison was impossible. An *intensity* comparison indicated the presence of 0.1275 mg. of tyrosine, or 125.5 per cent of the amount actually present.

Tryptophane does not interfere with the colorimetric determination of tyrosine unless the weight of tryptophane present exceeds the weight of the tyrosine. When the concentration of tryptophane is high, a yellow color is produced due to the interaction of tryptophane with *p*-phenyldiazonium sulfonate. A colorimetric comparison gives the *sum* of the yellow color due to tryptophane and the purple color due to tyrosine. The resultant reading is *high*.

Having shown that the discrepancy between my previous results and those obtained by Folin and his coworkers cannot be ascribed to an interference, by tryptophane, with the colorimetric process employed, it is next essential to examine the process that I used for possible errors.

The mode of procedure that I used in the tyrosine determinations reported in 1925 can be briefly outlined as follows: The acid protein hydrolysate is treated, in aqueous solution, with silver sulfate and barium hydroxide which gives a copious precipitate of

silver hydroxide that contains all of the histidine. The tyrosine was assumed to remain quantitatively in the filtrate. The filtrate is freed from silver and barium. It is then concentrated by evaporation on the steam bath. The residue is suspended in water, treated with mercuric acetate and acetic acid, and heated for 3 hours on the steam bath. Add sodium chloride to the cooled mixture and remove the solid by centrifugalization. Repeated experiments indicated that tyrosine appears quantitatively in the mercury precipitate. The latter is digested with sulfuric acid and freed from mercury by means of hydrogen sulfide.

There are, in this process, three possible sources of error. Some tyrosine may be carried down by the silver oxide. The mercury precipitation of tyrosine may not be quantitative. Even if tyrosine is quantitatively precipitated by mercury, some tyrosine may be lost by adsorption to the mercuric sulfide.

These possible sources of error have been subjected to experimental investigation.

Does Tyrosine Appear in the Silver Precipitate to Any Appreciable Extent?

Casein that had been hydrolyzed with sulfuric acid (2.0 gm.) was dissolved in 25 cc. of $\text{N H}_2\text{SO}_4$ and the solution transferred to a 1000 cc. flask with 50 cc. of water. Add 600 cc. of 0.8 per cent silver sulfate solution, mix thoroughly, and add a solution of 30 gm. of barium hydroxide in 150 cc. of warm water. Cool in an ice bath for 30 minutes. Centrifuge. This divides the material into two fractions: a silver precipitate which contains all of the histidine, and which may contain a small amount of tyrosine, and a silver filtrate which contains all or most of the tyrosine.

Silver Precipitate.—This is treated with 50 cc. of 2 $\text{N H}_2\text{SO}_4$ and mixed thoroughly to bring the silver complexes into solution as much as possible. Then add 40 cc. of N HCl and digest on the water bath. Adjust with a silver sulfate solution so that the liquid contains a trace of chloride. Remove all but a trace of sulfate with baryta. Filter. Evaporate to a small volume, transfer with water to a 10 cc. graduated cylinder, and dilute to exactly 10 cc.

The tyrosine content of this liquid was determined by use of the method of Folin and Looney⁷ as well as the Folin and Ciocal-

⁷ Folin, O., and Looney, J. M., *J. Biol. Chem.*, 1922, li, 421.

teu modification of the Millon reaction. The Folin and Looney reaction indicated the presence of 8.83 mg. of tyrosine; the Millon reaction, 2.96 mg. of tyrosine.⁸

From this it is quite obvious that a small, *but not negligible*, amount of tyrosine is carried down with histidine in the silver precipitate. This has been verified by working with pure solutions of tyrosine. It is also clear that the two colorimetric methods used give entirely different values. Of the two, the *Millon* reaction appears to be the more reliable. The merits of these methods are discussed later in this paper.

Tyrosine Is Quantitatively Precipitated as Tyrosino-Mercuric Chloride. Mercuric Sulfide Does Not Adsorb Tyrosine.

Tyrosine (10 mg.) is dissolved in 75 cc. of water. The solution is treated with 3.5 gm. of mercuric acetate and 1 cc. of glacial acetic acid and heated on the water bath for 3 hours. Cool, and add 7.5 gm. of sodium chloride. The mixture is placed in the ice chest overnight. It is then centrifuged, and the precipitate washed once with 5 cc. of water.

Mercury Precipitate.—This is digested on the steam bath for half an hour with 50 cc. of 2 N H_2SO_4 . The nearly clear solution is saturated with hydrogen sulfide. Filter after 24 hours, and wash the mercuric sulfide thoroughly with water. The filtrate is evaporated to a volume of about 50 cc. Transfer to a volumetric flask and dilute to 100 cc.

The tyrosine content of this liquid was determined by three methods:

	mg.
Folin and Looney	9.75
Millon	9.70
Hanke and Koessler	9.65

Mercury Filtrate.—This was treated with 25 cc. of N H_2SO_4 and saturated with hydrogen sulfide. Filter from HgS after 24 hours, evaporate the filtrate to a volume of approximately 25 cc., and dilute to exactly 50 cc.

⁸ Quantities of test solution were chosen, throughout this investigation, so that the color produced in the standard and that in the test solution were almost identical.

The tyrosine content of this liquid was determined by two methods:

Folin and Looney.....	mg. 0.56
Hanke and Koessler.....	0.417

A Millon determination could not be conducted on this fraction because of its high chloride content. Chlorides were removed from a portion of this liquid with silver sulfate. Sulfate was removed with barium. The solution finally obtained gave a Millon reaction that indicated the presence of 0.410 mg. of tyrosine for the entire test liquid.

An entirely similar experiment was conducted on 30 and on 100 mg. of tyrosine. The recovery of tyrosine in the mercury precipitate was practically quantitative in every case. Never more than 0.75 mg. of tyrosine remained in the filtrate. The total recovery was invariably quantitative. Experiments that have been conducted with synthetic mixtures of amino acids and with hydrolyzed proteins, to be reported later in this paper, all lead convincingly to the conclusion that tyrosine is quantitatively precipitated as tyrosino-mercuric chloride under the conditions specified above and that mercuric sulfide does not adsorb tyrosine. A possible 0.4 to 0.75 mg. that may remain in the filtrate is negligible in most cases.

Conclusions.

Tryptophane is completely destroyed when either egg albumin or casein is hydrolyzed with sulfuric acid or with hydrochloric acid. Tryptophane does not interfere with the Hanke-Koessler colorimetric process for estimating tyrosine unless its concentration exceeds that of the tyrosine. Tyrosine is, to some extent, carried down in the silver precipitate where we have, heretofore, sought only for histidine. Tyrosine can be removed quantitatively from a mixture of amino acids as tyrosino-mercuric chloride. Mercuric sulfide does not adsorb tyrosine.

There is, therefore, only one possible objection to the process for determining tyrosine that I described in 1925. The silver precipitate is apt to contain a small amount of tyrosine. The amount of tyrosine so adsorbed, and it probably is adsorbed, is far from sufficient, however, to explain the discrepancies between my

values and those obtained by Folin and his coworkers. This has led me to examine the processes employed by Folin and his coworkers. Experience with the phenol reagent of Folin and Looney and with the Millon reaction as modified by Folin and Ciocalteu, have led to some interesting observations which I shall present at this time.

*Phenol Reagent of Folin and Looney.*⁷—The method described by Folin and Looney for the estimation of tyrosine is very simple, but considerable experience is required before this method will give good checks. A precipitate is almost invariably obtained, and, unless this is removed completely, a slight cloudiness of the solution may lead to serious errors in judgment as to the color intensity of the solution. Folin and Ciocalteu⁵ have overcome the latter difficulty; but this work was reported just recently and some of my experiments date back over 2 years. I have found that the precipitate rarely forms when the determination is conducted as follows:

Mix 1 to 5 cc. of the test solution with 50 cc. of a 5 per cent solution of anhydrous sodium carbonate in a 100 cc. precision glass-stoppered graduated cylinder. The reagent (2 cc.) is blown forcefully into the alkaline solution from a 2 cc. volumetric pipette. Insert the stopper and invert the cylinder three or four times as rapidly as possible. Dilute with water to 100 cc. after 20 minutes and read after 30 minutes.

Accurate values are obtained with quantities of pure tyrosine ranging from 0.5 to 1.5 mg. It is practically always possible to arrange an experiment so that the amount of tyrosine falls within this range.

To obtain accurate readings, it is essential that the reagent be uniformly disseminated throughout the alkaline solution within 10 seconds. Folin and his coworkers have repeatedly called attention to the fact that the reagent is destroyed by the alkali. Since this is a reaction for which 30 minutes are required for a maximum color development, I obtained the impression that from 20 to 30 minutes are required for a complete destruction of the reagent. This is, however, not the case. The alkali appears to destroy the reagent within 15 seconds, which can be demonstrated as follows. Mix 2 cc. of reagent rapidly with 50 cc. of 5 per cent sodium carbonate. Add 1 mg. of tyrosine exactly 15 seconds after the reagent

begins to flow into the alkali. A blue color is either *not* obtained, or it is so faint as to be hardly discernible. From this it appears that if any reaction is going to occur it must get started within 15 seconds, or a complete destruction of the reagent will have occurred before it has had a chance to come in contact with all of the tyrosine.

Although the method of Folin and Looney gives perfectly accurate values with pure solutions of tyrosine, this is not always true when other amino acids are present.

A synthetic mixture was prepared, with Pfanstiehl products throughout, that contained 0.10 gm. each of the following amino acids: glycine, alanine, α -amino-*n*-valerianic acid, valine, leucine, isoleucine, aspartic acid, glutamic acid, phenylalanine, arginine and histidine. To this was added 0.0300 gm. of tyrosine. This mixture of amino acids will be referred to as Mixture 1.

Experiment A.—Mixture 1 was dissolved in $N H_2SO_4$, the solution transferred to a 100 cc. volumetric flask and diluted with $N H_2SO_4$ to 100 cc. The Folin and Looney reaction indicated the presence of 32.25 mg. of tyrosine, 107.5 per cent of the amount actually added. The Millon reaction of Folin and Ciocalteu indicated the presence of 30 mg. of tyrosine, 100 per cent of the amount actually added.

The Millon reaction gives the correct value in this case. The Folin and Looney value is 7.5 per cent high.

Experiment B.—Mixture 1 was mixed with 0.1000 gm. of cystine, the solid dissolved in $N H_2SO_4$, and diluted to 100 cc. The Folin and Looney reaction indicated the presence of 32.25 mg. of tyrosine, 107.5 per cent of the amount added. A white precipitate formed when the solution was heated with the Millon reagent, which turned red when the sodium nitrite was added. It was centrifuged to remove this precipitate. The color of the clear supernatant liquid indicated the presence of 22.5 mg. of tyrosine 75 per cent of the amount actually added.

Cystine has no effect upon the Folin and Looney value of a synthetic mixture of amino acids. The tyrosine value remained 7.5 per cent high. The Millon procedure of Folin and Ciocalteu cannot be used when cystine is present.

Experiment C.—Mixture 1 was treated with 75 cc. of water, 1 cc. of glacial acetic acid, and 3.5 gm. of mercuric acetate. Heat

on a water bath for 3 hours. Cool. Add 7.5 gm. of sodium chloride and allow to stand in the ice chest overnight. Proceed as described on page 593.

Mercury Precipitate.—The final volume of this fraction was 100 cc. The Folin and Looney procedure indicated the presence of 29.7 mg. of tyrosine in the entire 100 cc. sample, 99 per cent of the amount originally added. The Millon procedure indicated the presence of 29 mg. of tyrosine, or 97 per cent of the amount originally added.

Mercury Filtrate.—This was freed from chloride with silver sulfate and from mercury with hydrogen sulfide. Filter and evaporate the filtrate to a small volume. Finally dilute to 50 cc. The Folin and Looney procedure indicated the presence of 0.86 mg. of tyrosine in the total filtrate. The mercury precipitate from a synthetic mixture of amino acids *that does not contain cystine* contains practically all of the tyrosine. *Approximately the same amount of tyrosine is indicated by either the Folin and Looney or the Millon process.*

Experiment D.—Mixture 1 was mixed with 0.1000 gm. of cystine. Experiment C was then duplicated in every detail, excepting that the mercury filtrate was discarded.

Mercury Precipitate.—The final volume of this liquid was 100 cc. The Folin and Looney procedure indicated the presence of 52.6 mg. of tyrosine, or 175 per cent of the amount actually added.

This experiment has been repeated six times and values ranging from 143 to 175 per cent have been obtained. These values are, of course, entirely unreasonable. A perfectly accurate tyrosine figure can always be obtained with this material by mixing 3 cc. of the solution with 50 cc. of 5 per cent sodium carbonate and allowing the solution to stand for 2 hours before addition of the reagent.

3 cc. solution (slow process) = 16.6 mm.

1 mg. tyrosine = 15.0 "

= 30.1 mg. of tyrosine, or 100 per cent of the amount actually added.

Experiment E.—Experiment D indicates that when a mixture of amino acids containing cystine and tyrosine is boiled with mercuric acetate, products are formed that give a color with the Folin and Looney reagent, thus leading to an erroneous conception

as to the concentration of tyrosine. This error can be corrected by allowing the tyrosine-containing solution to remain in contact with the sodium carbonate for 2 hours before adding the reagent. Cystine appears to be the chief disturber. The next logical question is: Does the mercury treatment (1) produce a change in the cystine so that a color is obtained with it, (2) is it necessary to have present both cystine and tyrosine, or (3) must cystine be present together with tyrosine and with other amino acids?

Cystine does not give a color with the Folin and Looney reagent. Cystine that has been boiled with mercuric acetate and precipitated in the customary manner with sodium chloride, does not give a color with the Folin and Looney reagent.

A mixture of 50 mg. of cystine and 50 mg. of tyrosine was subjected to the mercury precipitation process. The final volume of the test liquid was 100 cc. The Folin and Looney procedure indicated the presence of 60 mg. of tyrosine, or 120 per cent of the amount actually added. This experiment was repeated four times. The results varied from 110 to 127 per cent. Normal values are always obtained with the "slow" process described above.

There can be no doubt, therefore, that cystine is a disturbing factor; but the effect is certainly not due to a direct action of the mercury-treated cystine on the reagent. The action seems, rather, to be due to an effect of the mercury-treated cystine on the tyrosine and this effect is intensified when other amino acids are present. A more complete study of this phenomena is now in progress.

I have used the "slow" process on a number of samples of hydrolyzed egg albumin and casein, but have never found any significant difference between the values so obtained and those obtained in the usual manner.

The Folin and Looney values are almost invariably higher than those obtained by the Millon process or by the Hanke-Koessler process. A startling deviation from the truth, as this is illustrated in the above experiment, is apt to make one just a little skeptical of the values obtained by this method.

*Millon Process of Folin and Ciocalteu.*⁷—This process gives accurate results with solutions of pure tyrosine. It cannot be used when cystine is present. Solutions that contain cystine give

a precipitate during the 15 minute period of heating (or even on standing at room temperature) and this precipitate contains tyrosine. Perfectly *clear* solutions are rarely obtained with protein hydrolysates—either alkaline or acid—when the process is carried out exactly as described by Folin and Ciocalteu. This slight turbidity registers as additional color and the reading obtained will depend largely upon the individual's interpretation as to how much of this shadow shall be disregarded. Readings that varied 20 per cent have been obtained by four persons in this laboratory. All of these persons are experienced in the use of a colorimeter.

The turbidity is not obtained until after the reaction liquid is diluted with water, and it can usually be avoided by diluting with N sulfuric acid. The clear, red solutions never do become turbid. The red color fades slowly, so that, after 18 hours a straw-colored solution remains.

It is, of course, unnecessary when one is working with *acid* hydrolysates to proceed exactly as noted by Folin and Ciocalteu because tryptophane is not present. For such determinations I have prepared a solution which embodies all of the various solutions advocated by Folin and Ciocalteu (to make the results comparable with those obtained when working with alkaline hydrolysates). This solution, which I shall subsequently refer to as the Millon reagent, is compounded as follows: Mix 80 cc. of a 15 per cent solution of mercuric sulfate in N sulfuric acid with 200 cc. of a 1.5 per cent solution of mercuric sulfate in $2 N$ sulfuric acid, and 120 cc. of $7 N$ sulfuric acid. The determinations on acid hydrolysates are then conducted as follows:

Mix 2 to 10 cc. of the acid hydrolysate with 20 cc. of the Millon reagent in a 50 cc. volumetric flask. Heat for 15 minutes in a boiling water bath. Cool. Add 1 cc. of a 2 per cent sodium nitrite solution. Mix. Dilute to 50 cc. with N sulfuric acid. A 2 mg. tyrosine standard is treated as above (the initial volume being the same as that of the test solution) and heated simultaneously with the test solution. Comparisons are made in a colorimeter.

Determination of Tyrosine in Casein and Egg Albumin.

1. Tyrosine (?) was determined in crude, acid hydrolysates as described above and in crude alkaline hydrolysates freed from tryptophane, by use of the method of Folin and Ciocalteu.

TABLE I.

Tyrosine Content of Casein and Egg Albumin.

Experiment No.	Sample No.	Protein used.	Age of protein.	Method used in drying protein.	Kind of hydrolysis.	Tyrosine (%) determined on crude hydrolysate.		Tyrosine content of Hg precipitate from crude acid hydrolysate.		Tyrosine content of Ag precipitate.		Tyrosine content of Hg precipitate from Ag filtrate.		Tyrosine content of Hg filtrate.	
						Rollin and Looney.	Millon.	Rollin and Looney.	per cent	Millon.	per cent	Rollin and Looney.	per cent	Rollin and Looney.	per cent
1	1	Casein.	hrs.	Vacuum.	H ₂ SO ₄	5.77	5.38	5.17	4.84					per cent	Millon.
2	1	"	9	"	"	5.56	5.36	4.90	4.85					0.142	per cent
3	1	"	9	"	"	5.60	5.40	5.0	4.84					0.075	per cent
4	1	"	9	"	"	5.36	5.36	4.85	4.84					Trace.	per cent
5	1	Casein.	9	Vacuum.	HCl	5.56	5.56	4.90	4.82					Trace.	Trace.
6	1	"	9	"	"	5.60	5.56	4.89	4.84					"	"
7	1	Casein.	9	Vacuum.	HCl	5.60	5.56			0.44	0.296	4.50	4.50	Trace.	Trace.
8	1	"	9	"	"	5.59	5.56			0.42	0.34	4.52	4.49	"	"
9	2	Casein.	Freshly pre-prepared.	Vacuum.	H ₂ SO ₄	6.1	5.86	5.56	5.48					Trace.	None.
10	2	"	"	"	"	6.2	5.90	5.60	5.45					"	"
11	2	Casein.	Freshly pre-prepared	4 days at 110°. Loss in weight = 3.0 mg. per gm. casein. Slightly yellow.	H ₂ SO ₄	5.86	5.77	5.28	5.28					Trace.	None.

2. Tyrosine was precipitated from the crude, acid hydrolysates as tyrosino-mercuric chloride as described above. The tyrosine content of the mercury precipitate and filtrate was determined by the Folin and Looney and by the Millon procedures. To employ the Millon procedure on the mercury filtrate it is, of course, essential to remove chlorides.⁹

3. Tyrosine was determined in the mercury precipitate fraction with the fractionation method described in my previous publication.¹ This involves a precipitation of the crude hydrolysate with Ag_2SO_4 and $\text{Ba}(\text{OH})_2$ and a subsequent preparation of tyrosino-mercuric chloride from the silver filtrate. A small amount of tyrosine appears in the silver precipitate. This fraction was, accordingly, tested for tyrosine by the Folin and Looney and by the Millon process.

The proteins used were prepared in this laboratory. Analyses were carried out on an old and on a freshly prepared sample of protein in each case. The effect of different methods of drying was also studied. The results are summarized in Table I.

Each experiment represents a separate *hydrolysis*, not merely a separate colorimetric determination. The figures recorded are the mean values ascertained from two to six separate colorimetric determinations.

DISCUSSION.

Of particular note in the first series of four experiments are the absolute uniformity of the Millon values on the mercury precipitate (4.84 per cent tyrosine), the divergence between the values obtained on the mercury precipitate and those obtained on the crude hydrolysate (from 0.5 to 0.9 per cent), and the almost complete absence of chromogenic substances in the mercury filtrate. Experiments 5 and 6 merely show that the results are identical with H_2SO_4 or with HCl hydrolysis. Experiments 7 and 8 are a verification of my previously reported results. The tyrosine

⁹ Chloride ion was removed from these filtrates with silver sulfate so that the Millon determination could be conducted. The Folin and Looney determination was carried out on the filtrate that *contained* sodium chloride and from which mercury had been removed with hydrogen sulfide, as well as on the portion of filtrate that had been freed from chloride with silver sulfate and from mercury with hydrogen sulfide.

content of this casein, as determined in the mercury precipitate after silver precipitation, is 4.5 per cent regardless of the method employed for the determination. This value is not quite accurate because a small amount of tyrosine is carried down with the silver precipitate. The Millon process gives 0.32 per cent tyrosine as the amount that is carried down with the silver oxide. If we add the amount of tyrosine recovered in the silver precipitate to that determined in the mercury precipitate we obtain 4.82 per cent tyrosine, which is identical with the value obtained when the crude hydrolysate is subjected *directly* to a mercury precipitation.

This is additional proof that the mercury precipitate contains all of the tyrosine. I feel justified in concluding that this sample of casein contained 4.8 to 4.85 per cent of tyrosine.

Experiments 9 and 10 were conducted on a freshly prepared sample of casein. This sample contains 5.45 per cent tyrosine which is 0.6 per cent more than was contained in the old sample. This difference may have been due to oxidation of tyrosine in the old sample or it may merely represent an inherent difference in the two preparations. It may be possible that casein from different cows or from the same cow at different times may vary slightly in composition. Then, too, the methods employed in preparing proteins are not absolutely certain to yield a chemical entity.

Experiments 11 and 12 show the effect of high temperature drying on the tyrosine content of casein. The samples lost only 3 mg. per gm. during a 4 day heating period. A slight coloration developed and the tyrosine content was reduced (from 5.45 to 5.25 per cent). The effect of heating is even more marked in the case of egg albumin (see Experiments 21 to 24). Folin and Ciocalteu obtained higher tyrosine values using the Millon procedure on an alkaline hydrolysate than had previously been obtained on acid hydrolysates with the Folin and Looney procedure. They interpret this higher value as being due to a more complete hydrolysis in alkaline solution. Experiments 13 to 15 show that this interpretation is incorrect.

The experiments on egg albumin are schematically identical with those on casein and the conclusions are identical. For details see Table I, Experiments 16 to 25.

CONCLUSIONS.

This method previously outlined by me for the determination of tyrosine in proteins gives results that are somewhat too low (0.3 to 0.6 per cent) because a small amount of tyrosine is carried down with the silver oxide in the silver precipitation process for the removal of histidine. This silver precipitation was an essential part of the process, at that time, because the colorimetric procedure for determining tyrosine also gives a color with histidine, and, unless histidine is removed, some of it appears in the mercury precipitate together with the tyrosine. The only other method of any merit for determining tyrosine that was available at that time was that of Folin and Looney. This method is not specific for tyrosine nor even for phenols. The experiments just described strengthen my belief that this method is not thoroughly reliable excepting on pure solutions of tyrosine. The modified Millon procedure of Folin and Ciocalteu appears, under proper conditions, to give very uniform results, and the tyrosine content as determined by their method is identical with that determined by the method of Hanke and Koessler. The Millon procedure has the advantage that it can be used to determine tyrosine in the presence of histidine.

A crude protein hydrolysate, either acid or alkaline, appears to contain something other than tyrosine or tryptophane that reacts with both the phenol reagent of Folin and Looney and the Millon reagent of Folin and Ciocalteu. This may be one substance, or there may be several; but whatever it is, it does not react in an identical manner with both reagents. Higher values are almost invariably obtained with the phenol reagent of Folin and Looney.

Tyrosine appears to be quantitatively precipitated, even from a mixture of amino acids and such other substances as might be present in a protein hydrolysate, when it is boiled in acetic acid solution with mercuric acetate and the mixture treated with sodium chloride. Removal of the mercury with hydrogen sulfide from either the mercury precipitate or filtrate does not lead to adsorption of tyrosine. Tyrosine determinations on the mercury precipitate give practically identical values regardless of which method of determining tyrosine is employed. Obviously, then, this precipitate, although it contains the tyrosine, does not contain

the other substances that react with either the Folin and Looney or the Millon reagent.

The filtrate from the mercury precipitate contains only a negligible amount of tyrosine, and, in most cases, it does not contain anything that reacts with either the Folin and Looney or with the Millon reagent. The chromogenic substances, other than tyrosine, disappear somehow in the course of the mercury treatment. The mercury precipitation process appears, therefore, to have a value that was not suspected at the time of its introduction. It not only separates the tyrosine from the bulk of other amino acids but it also removes substances, other than tyrosine, that react with both the phenol reagent of Folin and Looney and with the Millon reagent of Folin and Ciocalteu.

I believe that the tyrosine content of a protein can be accurately determined by conducting a colorimetric Millon determination on the mercury precipitate fraction from a crude, protein hydrolysate as outlined in this paper.

The method outlined in my previous publication can be recommended for use in such cases where it is desirable to determine both histidine and tyrosine in the same sample of material. It must be remembered, however, that some of the tyrosine appears in the silver precipitate where it can easily be determined with the Millon procedure of Folin and Ciocalteu. It might be well to call attention to the fact here that the colorimetric determination of histidine by the method of Koessler and Hanke in the silver precipitate, may, at times, be rendered difficult by the fact that the color obtained is slightly too yellow. This difficulty can almost invariably be overcome by subjecting the silver precipitate fraction to a precipitation with phosphotungstic acid as described in the paper by Hanke and Koessler.¹⁰ The color obtained with the phosphotungstic acid precipitate fraction is identical with that obtained with histidine.

Where enough material is available to make this possible, I would recommend determining histidine and tyrosine separately on two samples of hydrolysate, histidine to be determined in the phosphotungstic acid precipitate as outlined by Hanke and Koessler¹⁰ and tyrosine as follows:

¹⁰ Hanke, M. T., and Koessler, K. K., *J. Biol. Chem.*, 1920, xliii, 527.

1. Hydrolyze the protein with sulfuric acid and remove the sulfuric acid as outlined above.

2. The water-free residue finally obtained is transferred to a 250 cc. Florence flask with 75 cc. of water. Add 1 cc. of glacial acetic acid and 3.5 gm. of mercuric acetate and heat on the water bath for 3 hours. Cool. Add 7.5 gm. of sodium chloride, mix until dissolved, and cool in the refrigerator for 24 hours. Centrifuge. This divides the material into a mercury precipitate which contains all but negligible traces of the tyrosine as tyrosinomercuric chloride, and a filtrate which can be discarded.

3. The mercury precipitate is digested on the steam bath for 15 minutes in the centrifuge tube with 25 cc. of 2 N H_2SO_4 . Transfer the mixture to a 250 cc. flask with an additional 25 cc. of 2 N H_2SO_4 and 100 cc. of water. Saturate the mixture with hydrogen sulfide, stopper the flask, and allow to stand overnight at room temperature. Filter. Wash the mercuric sulfide thoroughly with water. Concentrate the filtrate to a volume of 50 cc. on the water bath, transfer to a volumetric flask, and dilute to 100 cc.

4. Determine tyrosine by use of the Millon procedure as outlined above.

The method just outlined gives perfect checks when it is employed on several samples of the same protein that have been dried and handled in an identical manner. The method used in drying the protein has a marked effect upon its tyrosine content. Samples that have been heated contain less tyrosine than those that have been dried *in vacuo*.

A preparation of casein, and one of egg albumin, that had been prepared several years ago, contained less tyrosine than did freshly prepared samples of the same protein. This may be interpreted as a loss due to ageing (oxidation ?); but it was not possible to prove this. This result may also be interpreted as being due to an original difference in the composition of the protein samples. It is not possible, at present, to make a definite statement with regard to this latter possibility because a uniform method for determining tyrosine has not yet been adopted and I do not believe that colorimetric determinations conducted on crude hydrolysates are an accurate index of the tyrosine content of the protein. A comparison of values obtained on crude hydrolysates, by different investigators using the same method, should,

however, be of some value. Let us, for example, examine the casein situation. Folin and Denis in 1912, using the phenol reagent on an *acid* hydrolysate (which was not hydrolyzed sufficiently but which should have been fairly free of tryptophane) determined a tyrosine content of 6.5 per cent. Looney refers to this as being the summation value of tyrosine and tryptophane; but this could hardly be true when the method of hydrolysis is considered. Folin and Looney, in 1922, using the phenol reagent on an *acid* or an *alkaline* hydrolysate determined a tyrosine content of 5.35 per cent. Folin and Ciocalteu, in 1927, using their Millon procedure (which should give results that are lower than those obtained by the phenol reagent) determined a tyrosine content of 6.37 and 6.55 per cent on two different samples of casein. My values on the *crude, acid* hydrolysate are 5.56 per cent with the Millon reagent on one sample of casein and 6.1 per cent (Folin and Looney) and 5.9 (Millon) on a second sample of casein. It appears, therefore, that every sample of casein investigated contained a different amount of tyrosine.

A perusal of the article by Folin and Ciocalteu⁵ is rather illuminating in this connection. They state, on page 646 in discussing edestin, "According to Folin and Looney, edestin should contain 1.40 per cent tryptophane and 5.7 per cent tyrosine. The latter figure must be due to some serious error, for the Folin-Looney process could not possibly give such a tyrosine figure for edestin. In his recent paper on the subject, Looney records the tyrosine of edestin as 4.58 per cent. . . . These figures are substantially identical with the values obtained by the methods described in this paper— . . . tyrosine 4.53 per cent." With all due respect for Dr. Folin's opinion in the matter, it is hard for me to believe that he and Looney could actually have made such an error because, although a slip is conceivable on one determination, they must surely have conducted these experiments in duplicate. I am inclined to believe that both figures are right and that the differences may represent either differences in the edestin or in the age or the method of drying the protein.

Folin and Looney determined 3.4 per cent tyrosine in gliadin. This was revised later by Looney to 3.04 per cent and then by Folin and Ciocalteu to 3.1 per cent.

These few examples, which could be multiplied considerably,

indicate strongly that the tyrosine content of proteins, as they are prepared for analysis, varies considerably for different samples of the same protein. Definite conclusions can hardly be drawn until a method is adopted for comparison that really determines tyrosine. I do not believe that the figures quoted here, all of which were obtained on crude hydrolysates, are an accurate index of the tyrosine content of the proteins.

The experimental data presented in this paper indicate that tyrosine can be quantitatively precipitated as tyrosino-mercuric chloride. The Millon method of Folin and Ciocalteu appears to give an accurate idea as to the tyrosine content of this precipitate. Determinations conducted on the mercury precipitate check perfectly for any given sample of protein but here, too, the tyrosine content is found to be different for different samples of the same protein. Thus one of my samples of casein contained 4.85 per cent tyrosine, the other 5.45 per cent.

SUMMARY.

1. The figures that I reported in 1925¹ for the tyrosine content of proteins are somewhat low (0.3 to 0.6 per cent). The low results are not due to the interference, by tryptophane, with the colorimetric process employed (as suggested by Looney) but to the fact that a small, but not negligible, amount of tyrosine is carried down by silver oxide and appears in the histidine fraction.

2. Tyrosine can be quantitatively precipitated from a crude protein hydrolysate as tyrosino-mercuric chloride.

3. The Millon procedure suggested by Folin and Ciocalteu gives very consistent, and I believe, accurate, tyrosine figures when the method is applied to the tyrosine fraction (that precipitated by mercuric acetate plus NaCl).

4. Crude protein hydrolysates appear to contain something other than tyrosine (or tryptophane) that reacts with both reagents. Higher values are almost invariably obtained with the phenol reagent. This substance disappears, somehow, in the course of the mercury precipitation process.

5. The tyrosine content of a given protein appears not to be absolutely constant. This may be due to an inherent difference in the tyrosine content, changes brought about by the method of

preparation, or changes produced by the process used in drying the sample for analysis. Old preparations of both casein and egg albumin contained less tyrosine than those that had been freshly prepared. Samples that have been heated at 110° contain less tyrosine than those that have been dried *in vacuo*.

I take pleasure in acknowledging the able assistance of Mr. C. M. Marberg.

A COLORIMETRIC METHOD FOR THE DETERMINATION OF INORGANIC PHOSPHATE IN BLOOD SERUM.

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The methods most generally used for the determination of phosphates in blood are based upon the reduction of phosphomolybdic acid to the blue molybdic oxide, the color of which is compared to a solution containing a known amount of phosphate. The first method based upon this principle was introduced by Taylor and Miller (1) and was later modified by Bell and Doisy (2), Briggs (3), Benedict and Theis (4), and Fiske and Subbarow (5). However, the conditions necessary to obtain quantitative results have to be very carefully adjusted. Thus Roe, Irish, and Boyd (6) found the following conditions to influence greatly the production of the blue color: the concentration of the molybdic acid; the concentration of the reducing agent; the time allowed for the completion of the reaction; the hydrogen ion concentration; the presence of salts; and the amount of phosphate in the sample to be analyzed.

It is evident that a method in which most of these obstacles could be eliminated would be worth while. Such a method is made possible by using a soluble uranium salt for isolating phosphate from solution, since phosphate combines quantitatively with uranium to produce an insoluble salt. A volumetric procedure based upon this principle for the determination of phosphate in urine has been in vogue for many years. Gibson and Estes (7) have modified the method for urine into a colorimetric procedure by adding a solution of uranium acetate of known strength in excess of the amount necessary to precipitate the phosphate completely, and after removing the insoluble uranium phosphate by filtration, determining the excess uranium acetate colorimetrically. Sato (8) applied this technique to feces and urine, but instead of deter-

mining the excess uranium, he washed the uranium phosphate on a filter paper until all soluble uranium was removed; he then redissolved the uranium phosphate and determined the amount of uranium colorimetrically.

I have applied this procedure with a number of modifications to the determination of phosphate in blood serum with very good results. There is very little interference from the substances normally present in blood and from the reagents used in the performance of the test. The only factor that has to be carefully adjusted is the acidity of the serum filtrate. This point will be discussed fully later.

Process.—The serum proteins are removed with trichloroacetic acid and the phosphate precipitated as uranium phosphate in an acetic acid medium which contains ammonium acetate. The precipitate is washed free from uranium acetate, redissolved in trichloroacetic acid, and the color developed with potassium ferrocyanide.

Method.

Place 2 cc. of serum in a test-tube, and add 4 cc. of distilled water and 4 cc. of 20 per cent trichloroacetic acid. Shake well, let stand for 10 minutes, and filter through an ashless filter paper. When the filtration is complete a little more than 5 cc. of filtrate is obtained. Place 5 cc. of the filtrate into a conical 15 cc. centrifuge tube which is graduated at 10 cc. Into a similar tube place 5 cc. of the standard phosphate solution. Add to each tube a drop of brom-thymol blue indicator solution. Add dilute ammonium hydroxide until the solution turns blue (pH 7.6). Add 0.3 cc. more of the dilute ammonium hydroxide and then add 5 per cent acetic acid solution until the color just turns yellow (pH 6.0). Add 1 cc. of uranium acetate solution and 2 cc. of 95 per cent alcohol. Let stand for 1 minute, shake well, and let stand for 15 minutes. Centrifuge for about 3 minutes at moderate speed and pour off the supernatant fluid, letting the tube drain for a minute by touching its mouth against a clean filter paper in order to remove the last drop. Add 5 cc. of 20 per cent alcohol and break up the precipitate with a thin glass rod; add 5 cc. more alcohol, washing off the rod. Centrifuge and pour off the supernatant fluid as before. Wash once more with 10 cc. of 20 per cent alco-

hol and pour off the supernatant fluid. All of the excess uranium acetate is thus completely removed. Dissolve the precipitate in 1 cc. of 20 per cent trichloroacetic acid. This is best done by holding the tube at its mouth between the thumb and the index finger of the right hand and repeatedly hitting the bottom of the tube against the palm of the left hand. Hold up against the light to make sure that the precipitate is completely dissolved. Add 5 cc. of water and 1 cc. of a 10 per cent solution of potassium ferrocyanide, and fill up with water to the 10 cc. mark. Mix, let stand for about 2 minutes, and compare in the colorimeter, setting the standard at 20 mm.

Calculation of Results.

$$\frac{S}{R} \times 5 = \text{mg. phosphorus in 100 cc. serum.}$$

S = reading of standard in mm.

R = " " unknown " "

Preparation of Reagents.

1. *Trichloroacetic Acid*.—This reagent is not obtained commercially in very pure state and may contain traces of phosphate. Since it boils at 195° without breaking down, it is advisable to purify it by distillation.

2. *Standard Phosphate Solution*.—Carefully weigh out 0.2193 gm. of pure dry KH_2PO_4 and dissolve in water in a 200 cc. volumetric flask, making up with water exactly to the mark. Place 20 cc. of this solution into a 500 cc. volumetric flask half filled with water, add 200 cc. of 20 per cent trichloroacetic acid, and make up to the 500 cc. mark with water. 5 cc. of this solution contain 0.05 mg. of phosphorus which is equivalent to 5 mg. per 100 cc.

3. *Ammonium Hydroxide*.—28 per cent NH_4OH diluted 1:5.

4. *Brom-Thymol Blue*.—0.5 per cent solution.

5. *Uranium Acetate Solution*.—Dissolve 20 gm. of uranium acetate in a liter flask half filled with water (do not heat). Add 30 cc. of glacial acetic acid and make up with water to a liter. Mix well until practically all of the uranium acetate is dissolved (some undissolved substance will remain). Let stand for 2 days and filter through an ashless filter paper. Any phosphate that may have been present in the uranium acetate is thus removed.

DISCUSSION.

The trichloroacetic acid in the serum filtrate and in the standard must be completely neutralized since uranium phosphate is highly soluble in this acid and no precipitation of phosphate will occur even in the presence of an excess of acetic acid. After the trichloroacetic acid is neutralized, an excess of ammonia is added in order to produce an excess of ammonium acetate upon neutralization with acetic acid. This is necessary because uranium phosphate is somewhat soluble in acetic acid and the ammonium acetate, by depressing the hydrogen ion, prevents the solution of uranium phosphate by the acetic acid.

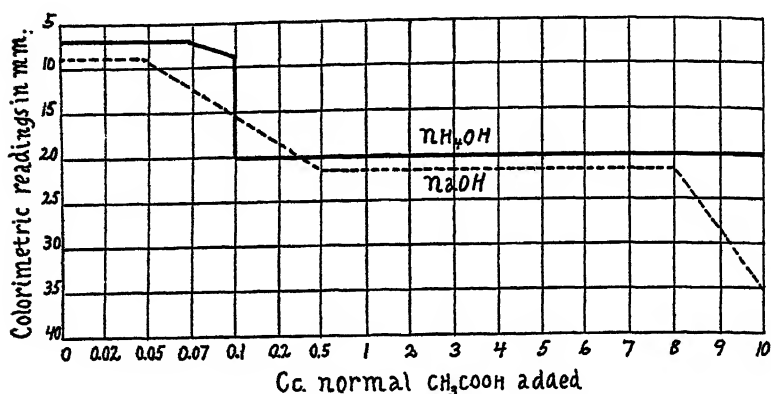


FIG. 1.

The amount of acetic acid added is of extreme importance. If the alkali is not completely neutralized some uranium will precipitate as $\text{NH}_4\text{UO}_2\text{O}_7$. When very little acid is present more phosphate is precipitated than when acetic acid is present in greater concentrations. If too much acetic acid is used precipitation of uranium phosphate will be hindered or entirely prevented even in the presence of ammonium acetate. The influence of acidity is shown by the curve (Fig. 1) in the accompanying diagram where colorimetric readings are plotted against acidity.

Thirty-four tubes were set up, each containing 5 cc. of the trichloroacetic acid phosphate standard. They were divided into two sets of seventeen tubes each. One set was neutralized with NH_4OH

NaOH and the other with N NH_4OH . Varying amounts of normal acetic acid were added to the tubes in each set and the amount of phosphorus determined colorimetrically as described in the method. One of the tubes showing a color of medium intensity was used as a standard, set in the colorimeter at 20 mm., and the rest of the tubes were read against it.

The curves obtained with the sodium and ammonium hydroxides show that the use of ammonia as a neutralizing agent is preferable to the use of sodium hydroxide. In the ammonia curve a maximum coloration occurs in the first four tubes. In this region the curve is very sharp. In the zone between 0.1 and 10 cc. of added normal acid there is no change in the intensity of the color produced. While the color in the first four tubes, where the acidity is low, is more than twice as deep as in the remaining tubes and should therefore be preferable in a colorimetric method, yet the disadvantage of the low margin of safety is too great to use low acidity. While the color is lighter in the remaining tubes, a straight line is obtained over a large range of acidity; thus by using higher acidity sources of error due to acidity are avoided. The mid-point of acidity on the straight line was chosen as the proper acidity to be used in the method.

In the beginning of the experiment NaOH was used as the neutralizing agent and it was thought that the deeper colors produced at the lower range of acidity were possibly due to some $NaUO_2O_7$ being precipitated since the NaOH, being a strong base, is easily dissociated and produces free OH^- ions, thus precipitating uranium oxide. That such was not the case was easily proved by the following experiments.

Experiment 1.—Controls containing trichloroacetic acid and no phosphate, when made just acid to brom-thymol blue without further addition of acid, repeatedly failed to produce a precipitate, even after standing overnight.

Experiment 2.—Added phosphate was completely recovered from solutions containing both very low and high acidity. Two sets of six tubes each were set up to which increasing amounts of phosphate were added. In one set the reaction was adjusted just acid to brom-thymol blue, while to the other set were added 5 cc. of N acetic acid. The amount of phosphorus was determined as described in the method. The first tube in each set was used as

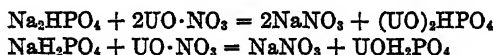
the standard and set in the colorimeter at 20 mm. The rest of the tubes were diluted with water to approach the intensity of the color of the respective standards. Complete recoveries of phosphate were obtained in each set in spite of the fact that the amount of precipitate, and consequently the intensity of the color, in the tubes of the low acidity were more than double in amount than in the tubes of the high acidity. The increase in color was in each case proportional to the amount of phosphate. This is shown by Table I and Fig. 2.

These experiments show definitely that at the different levels of acidity the precipitates consist of uranium phosphate and no uranium oxide is being precipitated. The difference in the amount of precipitate is probably due to different combinations of the uranium with the phosphate. A search in the literature revealed the

TABLE I.

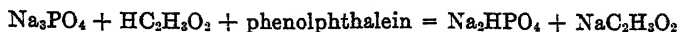
Tube No.	P	Dilution.	Low acidity.	High acidity.
	mg.			
1	0.05	None.	20	20
2	0.10	1:1	19.7	20.3
3	0.15	1:2	20.1	20.6
4	0.20	1:3	20.5	19.7
5	0.25	1:4	19.6	20.4
6	0.30	1:5	20.3	19.6

existence of some confusion as to the true nature of combination, some giving the formula UO_2HPO_4 , and some $(\text{UO})_2\text{HPO}_4$. Another possible combination is $(\text{UO}_2)_3(\text{PO}_4)_2$. Simon (9) says that with the dibasic phosphate 2 molecules of uranium combine with 1 molecule of phosphorus, while with the monobasic phosphate 1 molecule of uranium combines according to the following reactions:

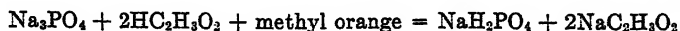


If these reactions were true, then it would seem that at low acidity the phosphate existed in the dibasic form, thus combining with twice as much uranium. That such is not the case can be easily proved. An aqueous solution of Na_3PO_4 was divided into

three equal parts. One portion was acidified with *N* acetic acid to phenolphthalein:



Another portion was neutralized with normal acetic acid to methyl orange:



No acid was added to the third portion. The three solutions were titrated with 2 per cent uranium acetate solution to which no

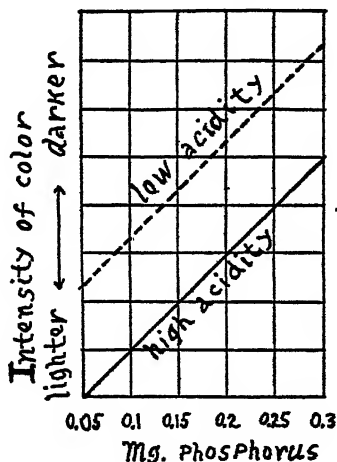


FIG. 2.

acetic acid was added, cochineal being used as an indicator. The following amounts of uranium acetate were necessary to combine with the three phosphate solutions:

Na_3PO_4	=	8.24	cc.	uranium acetate.
Na_2HPO_4	=	16.54	"	"
NaH_2PO_4	=	16.46	"	"

This shows that the mono- and dibasic phosphates combine in the same proportions with uranium acetate. In the case of the tribasic phosphate it seems probable that the precipitate was

uranium oxide, since Na_3PO_4 is easily hydrolyzed by water, producing a molecule of sodium hydroxide:



This question is being investigated at present, but since it has no direct bearing upon the method it is left out of this paper.

In the volumetric method for the determination of phosphate in urine by means of uranium, and in the colorimetric procedures of Gibson and Estes (7), and of Sato (8), the phosphate solution is first heated to about 95° before the uranium solution is added. In this method this was found unnecessary and even undesirable; better checks and better recoveries of phosphate were obtained in the cold. Moreover, when the solution is heated secondary calcium phosphate (CaHPO_4) may be precipitated.

TABLE II.

Sample No.	Added P per cc.	Serum plus P calculated per 100 cc. serum.		
		Recovered.	Calculated.	Per cent recovery.
	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	
1	None.	3.9		
2	0.02	5.8	5.7	98.3
3	0.04	8.1	7.9	102.5
4	0.06	9.8	9.9	99.0
5	0.08	11.6	11.9	97.5

The precipitate obtained on centrifugation is very gelatinous and packs tightly to the bottom of the tube so that no danger is encountered in losing any phosphate during the process of decantation.

The 20 per cent alcohol used for washing the precipitate prevents any possible solution of uranium phosphate, since it is absolutely insoluble in such a medium while uranium acetate is very soluble in it.

The stability of the color of uranium ferrocyanide depends largely upon the acidity. The higher the acidity the less stable the color. When left standing for some hours, the yellow color changes to blue and a precipitate settles out. This blue color is not proportional to the amount of uranium present but to the ferrocyanide.

The yellow color of uranium ferrocyanide develops immediately and will last without any change for at least half an hour.

Sato (8) used HCl for dissolving the uranium phosphate. The use of inorganic acids, however, was found to be detrimental to the results, since the presence even of minute traces of iron will produce a blue color with the ferrocyanide, and the color produced in absence of iron is not so good since the quality of the color varies with slight changes in the amount of HCl used. No such effect is produced with trichloroacetic acid.

Good recoveries of added phosphate were obtained from blood serum. A sample of serum which was found by this method to contain 3.9 mg. of phosphorus per 100 cc. was divided into five equal parts. No phosphate was added to the first part; to the other parts were added increasing amounts of phosphate. The results are shown in Table II.

SUMMARY.

A method is described for the determination of inorganic phosphate in blood serum. The proteins are removed with trichloroacetic acid; the filtrate is alkalized with ammonium hydroxide and reacidified with acetic acid, and the phosphate precipitated with uranium acetate. The uranium phosphate is washed free of uranium acetate with dilute alcohol. It is then redissolved in trichloroacetic acid, converted into uranium ferrocyanide, and is compared colorimetrically with a similarly treated solution containing a known amount of phosphorus. The color is proportional to the amount of uranium, thus to the amount of phosphorus.

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HEXOSE PHOSPHATES AND ALCOHOLIC FERMENTATION.

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(Received for publication, July 25, 1928.)

The participation of phosphoric acid in alcoholic fermentation and in the biological utilization of carbohydrates in general, is one of the most important discoveries made in connection with the study of these systems. Numerous attempts have been made to correlate this discovery with the many observations regarding the carbohydrate-phosphates which are formed in such biologic processes, but opinions are divergent as to the mechanism of the changes. Harden and Young¹ have emphasized the importance of the hexose diphosphate in fermentation while von Euler,² Meyerhof,³ and Kluyver and Struyk⁴ regard the formation of phosphoric esters of hexoses as a first step in the utilization of the carbohydrates; one of us⁵ has also expressed this same opinion. Neuberger,⁶ on the other hand, seems inclined to believe that the formation of phosphoric esters is only an incidental reaction and of little significance.

To the view that phospho-esters are intermediate products, there is the immediate objection that no known phospho-ester is

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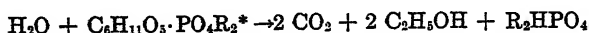
⁵ Raymond, A. L., *Proc. Nat. Acad. Sc.*, 1925, xi, 622.

⁶ Neuberger, C., *Biochem. Z.*, 1920, ciii, 320. Neuberger, C., and Gottschalk, A., *Biochem. Z.*, 1925, clxi, 244.

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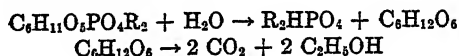
as rapidly fermented as is glucose. To dispose of this difficulty, Meyerhof³ has suggested that the esters as isolated are the stable forms while the true intermediate esters are unstable and are rapidly fermented.

In various discussions it has been assumed in general that the monophosphates are directly fermented as represented by the equation



An alternative explanation, however, and one which has not been sufficiently considered, is that the phospho-esters that have been studied may be first hydrolyzed by an enzyme to a hexose, and the hexose then fermented.

In equation form this would be



The object of the present study was to test this possibility. Our two aims were: first, to see if hydrolysis of phospho-esters could take place in the absence of fermentation; and second, to see if the rate of such hydrolysis was sufficient to supply hexose for observed fermentation.

The method which we selected involved the use of a zymin preparation which showed an induction period. The rate of hydrolysis of various phospho-esters was determined during this period and in parallel experiments the rate of fermentation by the same zymin was measured after minimal quantities of hexose diphosphate had been added to eliminate the induction period and induce immediate fermentation. The rate of such fermentation was then compared with the previously determined rate of hydrolysis.

Phospho-Esters and Induction Period of Glucose.

As a preliminary, it was necessary to examine the effect of the various natural and synthetic phospho-esters on the induction period. The relationship between induction time and concentration was determined for crude hexose diphosphate and it was found

* R is used to indicate either metal or hydrogen ion and in any proportion.

that at 0.005 mols per liter the fermentation started almost instantly (Fig. 2). To see whether some impurity in the diphosphate might be responsible for the decreased induction time, the strychnine salt was subjected to repeated recrystallization. The purified material was somewhat less effective than the crude, but after four recrystallizations the activity was not greatly changed by four further recrystallizations. This would indicate that if an impurity is responsible, it is not easily removed. The most effective of the monophosphates was the Neuberg ester prepared by partial hydrolysis of the diphosphate. The Robison ester was next in activity followed by dihydroxyacetone phosphate and 1-fructose phosphate (Fig. 2). The other synthetic esters employed, namely glucose, fructose, 3-glucose, and 3-fructose monophosphates, were without effect. In no case were any of the monoesters comparable in activity with the diphosphate.

These results are similar to those recently reported by Mayer⁷ in those cases in which identical esters were used.

Hydrolysis and Fermentation of Phospho-Esters.—Having established the fact that the monophosphates did not completely suppress the induction period, we were in a position to study their possible hydrolysis during that time.

Harden⁸ suggested many years ago that the effect of arsenates on fermentation was to increase the hydrolysis of the diphosphate. To see whether this also applied to the monophosphates the experiments were all performed in duplicate and to one of each pair arsenate was added.

By employing the colorimetric method of Kuttner and Cohen,⁹ which we found to be specific for inorganic phosphate, the changes in the latter were measured during the induction. It was found that some organic phosphate present in the zymine was hydrolyzed during the induction period, and that the synthetic esters showed a somewhat greater production of inorganic phosphate. In the case of the esters, arsenate caused but little acceleration of the hydrolysis and was sometimes inhibiting (Table I).

The rates of fermentation of the identical esters were next meas-

⁷ Mayer, P., *Biochem. Z.*, 1927, clxxvi, 313.

⁸ Harden, A., and Young, W. J., *Proc. Chem. Soc.*, 1906, xxii, 283; *Proc. Roy. Soc. London, Series B*, 1911, lxxxiii, 451.

⁹ Kuttner, T., and Cohen, H. R., *J. Biol. Chem.*, 1927, lxxv, 517.

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ured after addition of a small amount of diphosphate to abolish the induction period (Table II). If the values be compared with those given in Table I, it will be observed that in every case the dephosphorylation exceeded the carbon dioxide production when both were expressed in mols per liter. We may write

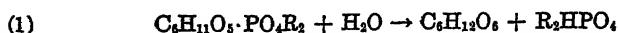


TABLE I.
Inorganic Phosphate (mM per Liter).

Time, min.	Blank.		Glucose phosphate.		3-Glucose phosphate.		Fructose phosphate.		1-Fructose phosphate.		3-Fructose phosphate.	
	Control.	Arsenate.	Control.	Arsenate.	Control.	Arsenate.	Control.	Arsenate.	Control.	Arsenate.	Control.	Arsenate.
Concentration (measured).												
10	18.1	22.5	19.3	22.1	19.6	22.9	23.2	27.2	20.2	22.9	25.7	26.9
30	22.9	28.7	26.0	28.6	27.8	30.9	30.7	34.4	26.7	30.1	32.8	35.4
50	29.0	36.7	32.4	34.0	35.6	38.2	38.2	42.4	34.9	38.8	39.1	40.9
70	29.3	36.6	34.4	36.7	37.0	39.1	36.5	43.4	37.3	39.7	40.0	42.0
100	30.1	36.7	29.0	36.6	34.0	40.0	33.5	42.7	31.6	40.7	37.3	41.4
Increase (calculated).												
30	4.8	6.2	6.7	6.5	8.2	8.0	7.5	7.2	6.5	7.2	7.1	8.5
50	10.9	14.2	13.1	11.9	16.0	15.3	15.0	15.2	14.7	15.9	13.4	14.0
70	11.2	14.1	15.1	14.6	17.4	16.2		16.2	17.1	16.8	14.3	15.1
100		14.2		14.5		17.1		15.5		17.8		14.5

The figures in bold face type indicate that carbon dioxide production had started and the corresponding differences are therefore omitted.

and either



or



If this mechanism is correct then the measurements of the dephosphorylation establish a minimum for the rate of reaction (1) and therefore of hexose formation. The formation of carbon dioxide by reaction (2) requires only 1 mol of hexose for each mol

of carbon dioxide formed and by (3) only 0.5 mol of hexose. It is thus evident that on either basis the rate of dephosphorylation is more than enough to account for the observed fermentation. These esters are therefore most probably first hydrolyzed to the free sugars and the latter then ferment. One point should be added; namely, that the rate of carbon dioxide production, though recorded for only the first $1\frac{1}{2}$ hours, actually showed a steady decrease with time, so that for each ester the initial rate of dephosphorylation exceeded the maximum rate of fermentation.

TABLE II.
Carbon Dioxide.

Time, min.	Blank.		Glucose phosphate.		3-Glucose phosphate.		Fructose phosphate.		1-Fructose phosphate.		3-Fructose phosphate.	
	Control.	Arsenate.	Control.	Arsenate.	Control.	Arsenate.	Control.	Arsenate.	Control.	Arsenate.	Control.	Arsenate.
Cc. per 10 cc. (measured).												
10	0.08	0.14	0.21	0.24	0.09	0.35	0.35	0.24	0.09	0.20	0.09	0.24
30	0.56	0.96	0.64	1.27	0.72	1.24	0.80	1.24	0.55	0.74	0.56	1.13
50	1.17	1.84	1.04	1.87	1.50	1.99	1.30	1.93	1.14	0.83	1.17	1.98
70	1.74	2.51	1.42	2.44	2.24	2.64	1.79	2.47	1.73	1.09	1.81	2.73
100	2.61	2.94	2.03	3.27	3.34	3.48	2.55	3.16	2.57	2.88	2.68	3.21
Increase, mm per liter (calculated).												
30	2.1	3.7	1.9	4.6	2.8	4.0	2.0	4.5	2.1	2.4	2.1	4.0
50	4.9	7.6	3.7	7.3	6.3	7.3	4.2	7.5	4.7	2.8	4.8	7.8
70	7.4	10.6	5.4	9.8	9.6	10.2	6.4	9.9	7.3	4.0	7.7	11.1
100	11.3	12.5	8.1	13.5	14.5	14.0	9.8	13.0	11.1	12.0	11.6	13.3

That the rates of fermentation and of hydrolysis were not always parallel seems to be due to inhibition of the fermentation by the esters. Such inhibition is quite evident in those cases in which the rate of fermentation of the ester was less than that of the blank.

Similar studies were made on the Robison and Neuberg esters except that in their case the induction period in the absence of arsenate was too short to be useful, and so this series was omitted. In the presence of arsenate, however, there was a period of no

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carbon dioxide production and the rate of dephosphorylation during this time was approximately equal to the fermentation observed in the presence of diphosphate (Table III). Slightly more than 1 mol of CO_2 was formed per mol of liberated hexose, but never as much as the 2 mols permitted by equation (3) above.

In view of these observations, first, that hydrolysis of all the monophosphates is constantly in progress, and second, that the rate of hydrolysis is sufficient to account for the observed carbon dioxide production, it seems justifiable to conclude that all these monophosphates are first hydrolyzed to the hexoses and that the latter then ferment.

TABLE III.

Production of Inorganic Phosphate and Carbon Dioxide. (mm per Liter).

Time, min.	Phosphate.			Carbon dioxide.		
	Blank.	Robison.	Neuberg.	Blank.	Robison.	Neuberg.
8						
19	3.2	5.5	3.9	2.5	7.1	7.1
32	7.2	10.2	9.4	5.6	14.6	14.5
47	13.3	17.2	15.9	9.0	19.6	18.9
64		19.9		11.7	23.7	22.9

As Harden¹ drew the same conclusion many years ago in regard to the method of utilization of the diphosphate this appears to be a general process. Any phospho-ester which is a true intermediate in the fermentation mechanism should be of such nature that its rate of fermentation greatly exceeds its rate of hydrolysis. This is certainly not the case for the monophosphates which we have studied.

Another point of interest which was derived from the data was the effect of the structure of the esters on their rate of enzymic utilization. Of the synthetic esters it was found that the 3-glucose phosphate exhibited the highest rate of dephosphorylation and fermentation, and that the 1-fructose ester was next. A similar result was obtained by Nodzu¹⁰ although he measured only rates of fermentation. It is interesting, in view of the fact that all of

¹⁰ Nodzu, R., *J. Biochem.*, 1926, vi, 31.

the rates found by him were greater than ours, that the relative order should be the same.

Also in conformity with previous findings, our data show that the Robison and Neuberg esters are more rapidly utilized than are the synthetic derivatives. Since the observed rates bear no relation to the stability of the esters toward inorganic reagents, it is evident that the phosphatase activity is intimately related to the structure of the ester.

Concentration Effect and Arsenate.—If it be true, as concluded above, that the rate of fermentation is determined by the rate of dephosphorylation, then the carbon dioxide production may be used as a measure of the hydrolytic activity in those cases in which the hydrolytic dephosphorylation is obscured or confused by the fermentation. We therefore examined the effect of arsenate and of varying concentration of the esters on the carbon dioxide production from the Robison, Neuberg, and Harden esters. The interesting points observed (Figs. 3 to 5) were that: (1) arsenate greatly accelerated the fermentation of all three esters; (2) increasing the concentration of the esters from 0.015 to 0.06 molal increased the rate of fermentation—most for the Neuberg and least for the diphosphate; (3) increasing the concentration of the ester increased the arsenate effect in the case of Neuberg and Robison esters but caused a preliminary depression with the diphosphate.

EXPERIMENTAL.

Methods.

Fermentation.—The carbon dioxide determinations were made in an apparatus in which tubes placed in a thermostat at 25.0° were constantly and vigorously shaken to cause rapid liberation of the carbon dioxide. At the start the tubes were partially evacuated and the increase in pressure was measured at constant volume. The apparatus was so arranged that six determinations could be made simultaneously.

Phosphate Determination.—The colorimetric method of Kuttner and Cohen⁹ was used, as preliminary experiments had shown that of all the organic phosphates employed, none showed more coloration than would result from the trace of inorganic phosphate which

they might have contained as impurity. It was therefore assumed that the method could be used to estimate inorganic phosphate selectively in these studies.

0.50 cc. of the fermenting mixture (shaken constantly under reduced pressure so as to be CO_2 -free) was removed with an Ostwald pipette and added to 12.0 cc. of ice-cold 0.8 per cent trichloroacetic acid. After the contents were mixed by being inverted two or three times, the tube was allowed to stand 5 minutes in an ice water mixture and then filtered through ash-free filter paper. Of the filtrate 0.50 cc. was used for analysis. A calibration curve was prepared to avoid errors resulting from the fact that the color produced is not always a straight line function of the concentration. A further modification introduced was that the amount of stannous chloride recommended in the original article was increased by 50 per cent. With these changes the method gave results reproducible to 1 to 2 per cent and accurate (as tried on fermentation mixtures containing known amounts of inorganic phosphate) to the same figure.

Preparation of Phospho-Ester.—The details of preparation of the synthetic esters will be described later; at this point it need only be mentioned that the general methods were those of Levene and Meyer,¹¹ Komatsu and Nodzu,¹² and Nodzu.¹⁰ Two preparations, probably mixtures of isomers, were obtained by direct phosphorylation of glucose and fructose, and three which should be single isomers, by phosphorylation of the diacetone hexoses and subsequent removal of the acetone groups. The most recent views of the structure of the diacetone compounds would ascribe carbon atom (3) to the phosphate from diacetone glucose, atom (3) to that from α -diacetone fructose, and atom (1) to that from β -diacetone fructose. These positions will be used below to describe the three esters, while the products obtained by direct phosphorylation will be called simply glucose and fructose phosphates.

The Neuberg ester was prepared by partial hydrolysis of the diphosphate with hydrochloric acid as described by Meyerhof.⁸

The Robison ester was prepared by the action of live yeast on

¹¹ Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, 1922, liii, 431.

¹² Komatsu, S., and Nodzu, R., *Mem. Coll. Sc., Kyoto Imp. Univ.*, 1924, vii, series A, 377.

glucose and sodium phosphate in the presence of toluene. An excess of glucose was used, and the phosphate was added slowly to maintain an optimum concentration and was followed by frequent colorimetric analyses. While the fermentation was still proceeding vigorously it was interrupted by adding trichloroacetic acid and the mixture was centrifuged. The diphosphate and inorganic phosphate were precipitated by adding a solution of barium chloride equivalent to the phosphate used, and then barium hydroxide to pH 9. After being centrifuged, the solution was clarified with charcoal and concentrated under reduced pressure to a small volume. The monophosphate was precipitated by adding an equal volume of 95 per cent alcohol and purified by repeated solution and precipitation. It analyzed well for a hexose monophosphate but showed a trace of nitrogen. It was identified by formation of the characteristic osazone described by Robison¹³ decomposing at 137°.

Dihydroxyacetone phosphate was prepared by phosphorylation of the triose with phosphorus oxychloride in pyridine solution and isolation as the barium salt.

The hexose diphosphate employed was candiolin¹⁴ and except where noted was used without further purification as the analysis was almost perfect. The calcium salt was converted to the sodium salt by suspending it in a solution of an excess of sodium carbonate and shaking in a machine for several hours in the cold. After filtration, the excess of carbonate was destroyed with acid and the solution analyzed for total and for inorganic phosphorus. The organic phosphorus was determined by difference and the solution diluted to the desired strength.

Phospho-Esters and Induction Period of Glucose.

A standard mixture prepared with the following substances was used: 0.4 gm. of glucose, 0.4 gm. of zymine, 0.4 cc. of 0.33 M sodium phosphate buffer and 3.6 cc. of H₂O (pH 6.4), and 4 drops of toluene.

A part of the water was replaced by a solution of the desired

¹³ Harden, A., and Robison, R., *Proc. Chem. Soc.*, 1914, xxx, 16. Robison, R., *Biochem. J.*, 1922, xvi, 809.

¹⁴ We wish to take this opportunity to thank the Winthrop Chemical Company, Inc., for giving us the candiolin for these and other experiments.

ester. All the esters were used in form of their sodium salts and were acidified to pH 6.4.

Hexose diphosphate concentrations up to 0.005 molal were employed. The data were plotted and the rate of carbon dioxide production was determined graphically. These rates were then plotted as a function of the time and are given in Fig. 1. It will be seen that when once started, the course of carbon dioxide pro-

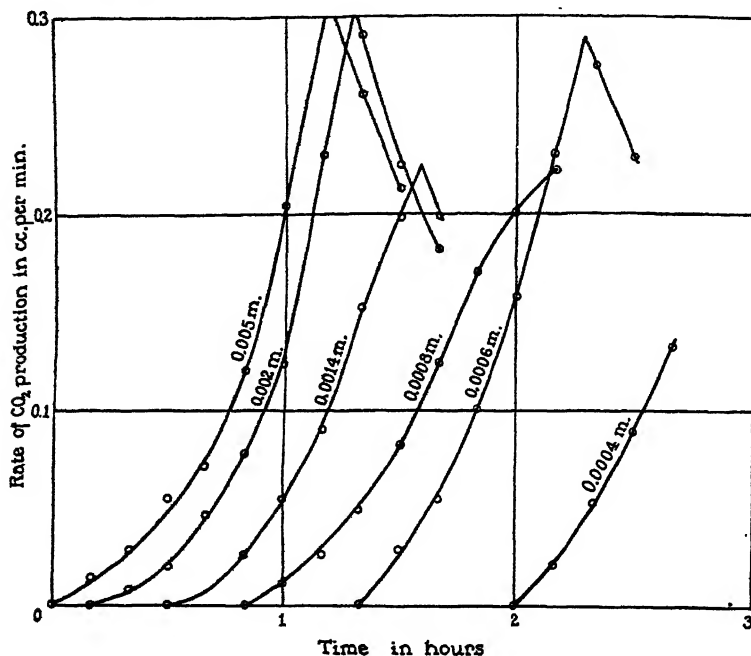


FIG. 1. Effect of hexose diphosphate on induction time and rate of fermentation.

duction was always the same, different amounts of the diphosphate producing a displacement of the complete curve along the time axis. In some few cases it was found that the slope did not increase quite so rapidly as in these experiments, but in general it was sufficient to consider only the time displacement of the curves as they were identical in other respects within the limits of experimental error. The relationship between the time of starting and the concentration of the diphosphate is shown in Fig. 2.

The effect of crude hexose diphosphate on the induction period having been thus established, the question remained as to whether pure hexose diphosphate was actually responsible for this decreased lag, or whether the activating substance was some adhering impurity. To test this, two samples of the strychnine salt were prepared and one was crystallized four and the other eight times. These were converted to the barium and then to the sodium salt and finally acidified to pH 6.2 to 6.4. For comparison with the diphosphate the results are included in Fig. 2. It will be observed

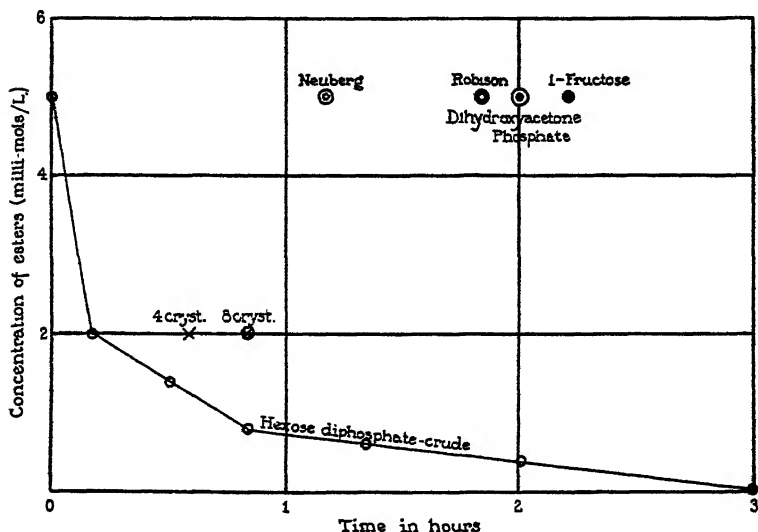


FIG. 2. Effect of hexose diphosphate and of several phospho-esters on induction period.

that the treatment in each case caused an increase in the induction period, but that the material which had been eight times crystallized was only slightly less effective than the four.

In order to see whether other organic phosphates would produce similar results, the hexose diphosphate in the above mixture was replaced by several synthetic and natural monophosphates.

It was found that the glucose, 3-glucose, fructose, and 3-fructose monophosphates produced no effect on the induction period while the 1-fructose, Neuberg and Robison monophosphates each caused

a decrease in the time of induction. The results are included in Fig. 2 to compare them with the diphosphate. It will be seen that none of the substances tested has an effect of the same order of magnitude as that of the hexose diphosphate.

Inasmuch as a triose monophosphate has the same composition as a hexose diphosphate, such a substance might easily be an undetected impurity of the crude diphosphate. The only avail-

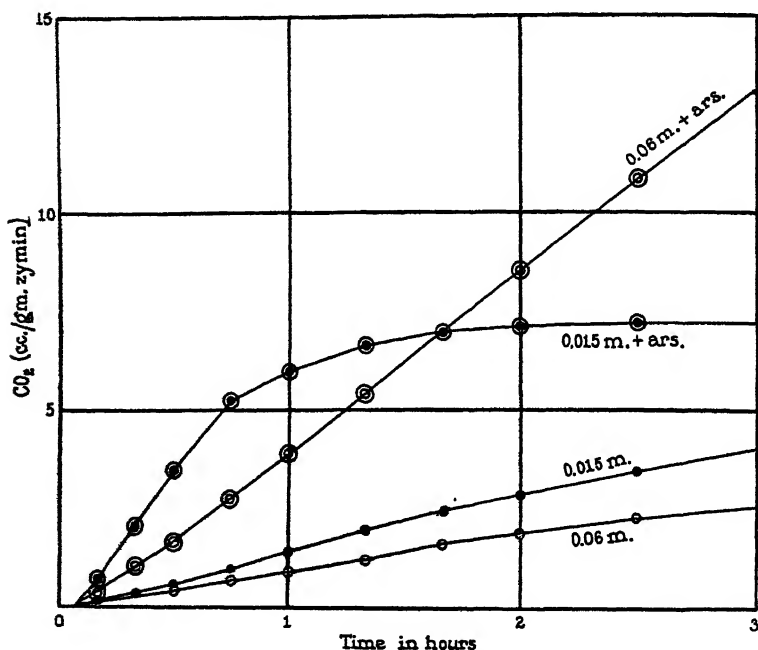


FIG. 3. Fermentation of hexose diphosphate in presence and absence of 0.004 molal arsenate. Ester 0.015 and 0.06 molal.

able triose was dihydroxyacetone and therefore we tested both it and its synthetically prepared phosphate. The triose produced no lowering of the induction period, and the effect of the phosphate was not comparable with that of the hexose diphosphate as may be seen in Fig. 2.

Hydrolysis and Fermentation of Phospho-Esters.—The fermentation mixtures consisted of a 10 per cent suspension of zymin in a 0.06 molal solution of the desired ester, to which toluene was

added in each case. The phosphate determinations were made by the colorimetric procedure previously described. As the duration of the induction period varied with the different esters employed, the moment at which carbon dioxide production commenced was determined on each sample and all values secured after this time were discarded. Where arsenate was employed its concentration was 0.004 molal, as preliminary experiments had established the

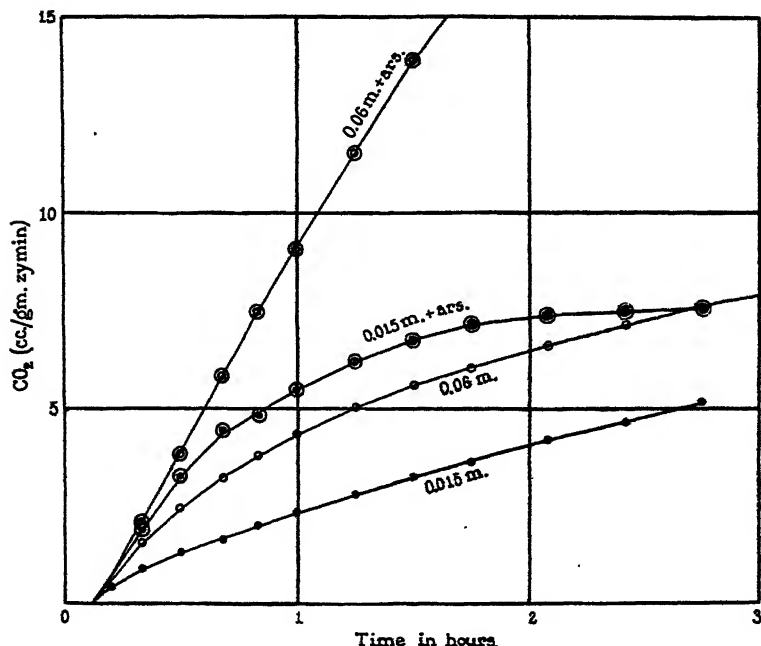


FIG. 4. Fermentation of Robison ester in presence and absence of 0.004 molal arsenate. Ester 0.015 and 0.06 molal.

fact that greater concentrations resulted in an inhibition of fermentation. All solutions employed were adjusted to pH 6.4 and this was also the pH of the final mixtures. The results of the various experiments on the synthetic esters are given in Table I.

Fermentation mixtures like those above were next prepared, except that hexose diphosphate was added to the extent of 0.004 mols per liter to eliminate the induction and on these mixtures the carbon dioxide production was measured by the method described.

The data were plotted and by interpolation the amounts of carbon dioxide formed at the above time intervals were determined. These results were converted to equivalent units—in this case to mm of CO_2 formed per liter of fermentation mixture—and are given in Table II.

On attempting to extend the experiments to the Robison and Neuberg esters it was found that they both lowered the time lag

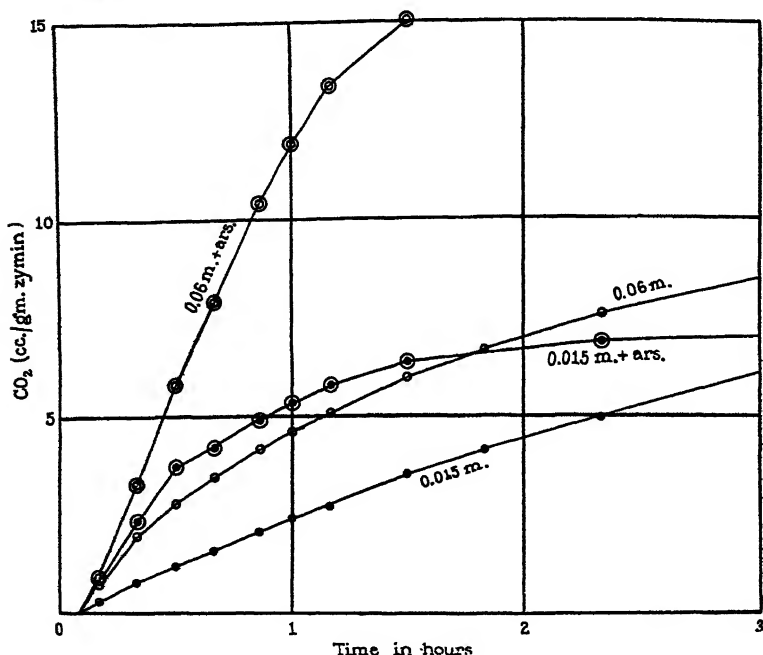


FIG. 5. Fermentation of Neuberg ester in presence and absence of 0.004 molal arsenate. Ester 0.015 and 0.06 molal.

very greatly at a concentration of 0.06 mols per liter. However, by reducing the concentration, and by adding arsenate which increases the induction period, it was found possible to make the desired determinations. In Table III are given the carbon dioxide production and the increase of inorganic phosphate during the induction period, as determined for 0.012 molal concentrations of the esters in the presence of 0.004 molal arsenate.

Concentration and Arsenate Effect.—To see how concentration affected the rate of hydrolysis, and hence of fermentation, of the Robison, Neuberg, and Harden esters, the rate of carbon dioxide production was measured in the absence and presence of 0.004 molal arsenate. The esters were 0.015 and 0.06 molal, the zymin was 1.0 gm. per 10 cc. of fermenting mixture, and toluene was added as antiseptic. The results are given in Figs. 3 to 5.

SUMMARY.

1. The effect of a number of synthetic and natural phosphoesters on the induction period of glucose with zymin was determined. The hexose diphosphate greatly exceeded all the others in abolishing the induction. It was not found possible to remove this property of the diphosphate by repeated recrystallization as the strychnine salt.

2. The rate of hydrolysis of the esters was measured during the induction period and found to exceed the carbon dioxide production observed when the induction period was removed with added diphosphate. This is interpreted as indicating that all the observed esters are hydrolyzed to a free hexose which is then fermented.

3. The rate of hydrolysis, and hence of fermentation, depended upon the configuration of the ester. The natural products were more rapidly utilized than the synthetic.

4. The effect of concentration and of arsenate on the fermentation of the Neuberg, Robison, and Harden esters was determined. Arsenate greatly increased the fermentation rate of all three esters, and increased concentration of ester produced a similar but smaller effect.

5. Addition of arsenate produced but little increase in the rate of hydrolysis or fermentation of the synthetic esters.

COZYMASE. ITS RELATION TO PHOSPHATASE ACTIVITY.

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(Received for publication, July 24, 1928.)

Although cozymase, the complement enzyme of alcoholic fermentation, is considered most often in connection with carbon dioxide production, nevertheless, several additional functions have been ascribed to it. Thus it has been stated to be a necessary complement of the phosphate-esterifying enzyme, phosphatase (Neuberg,¹ von Euler²), of aldo-mutase (von Euler^{3,4}), of methyl-glyoxalase (Gottschalk⁵), and of oxido-reductase (von Euler^{4,6}). This last function has been contested, however (Lebedev⁷), and the cozymase has been found to be not necessary for the action of carboxylase (Harden,⁸ Neuberg⁹). Also it has been found that cozymase-free zymin would hydrolyze hexose diphosphate (Harden and Young^{10,11}).

In connection with work on the phosphate esters, we had occa-

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¹ Neuberg, C., and Gottschalk, A., *Biochem. Z.*, 1925, clxi, 244.

² von Euler, H., *Z. physiol. Chem.*, 1924, cxxxix, 15.

³ von Euler, H., and Nilsson, R., *Z. physiol. Chem.*, 1926, clxii, 72.

⁴ von Euler, H., *Svensk Kem. Tidskr.*, 1926, xxxviii, 353; *Ergebn. Physiol.*, 1928, xxvi, 553.

⁵ Gottschalk, A., *Z. physiol. Chem.*, 1928, clxxvi, 314.

⁶ von Euler, H., and Brunius, E., *Z. physiol. Chem.*, 1928, clxxv, 52.

⁷ Lebedev, A., *Z. physiol. Chem.*, 1927, clxxii, 255.

⁸ Harden, A., *Biochem. J.*, 1913, xi, 64.

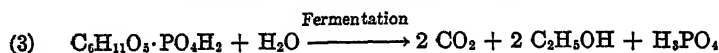
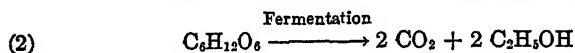
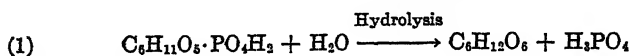
⁹ Neuberg, C., and Rosenthal, P., *Biochem. Z.*, 1913, li, 128. Neuberg, C., *Biochem. Z.*, 1915, lvi, 497.

¹⁰ Harden, A., and Young, W. J., *Proc. Roy. Soc. London, Series B*, 1910, lxxxii, 321.

¹¹ Harden, A., and Young, W. J., *Proc. Roy. Soc. London, Series B*, 1908, lxxx, 299.

sion to study the rôle of cozymase in the phosphatase mechanism. As the studies indicate that the coenzyme is intimately involved in phosphatase action, it seems desirable to present a brief report on the results obtained.

The view that the hexose diphosphate is first hydrolyzed and then fermented was advanced long ago by Harden.¹¹ Recent work in this laboratory indicated that this was also the case with all the monophosphates, synthetic and natural, which were accessible to us.¹² Thus, the accumulation of inorganic phosphate in an enzymic fermentation of these esters is evidence, not of fermentation but of hydrolysis, the sequence being (1) and (2) and not (3).



Assuming that this mechanism is correct, a study of the increase in inorganic phosphate affords a means of examining the action of the phosphatase. However, it must be remembered that fermentation is usually in progress accompanied by esterification, and that inorganic phosphate is thus being constantly used up. Any figures on the appearance of inorganic phosphate are therefore too low except when esterification is excluded.

The esterification may be decreased, however, and the phosphatase activity simultaneously increased, by addition of arsenates to the fermentation mixtures (Neuberg,^{13,14} Harden and Young¹⁵). In our first experiments we therefore used arsenate, and studied the changes of phosphatase activity produced by removal of the coenzyme. Acetone-dried yeast was employed and was freed of coenzyme by repeated washing. After such treatment the residue was entirely inactive as far as carbon dioxide production from glucose was concerned.

¹² Raymond, A. L., and Levene, P. A., *J. Biol. Chem.*, 1928, lxxix, 621.

¹³ Neuberg, C., and Leibowitz, J., *Biochem. Z.*, 1927, xcxi, 460.

¹⁴ Neuberg, C., and Kobel, M., *Biochem. Z.*, 1926, clxxiv, 493.

¹⁵ Harden, A., and Young, W. J., *Proc. Chem. Soc.*, 1906, xxii, 283; *Proc. Roy. Soc. London, Series B*, 1911, lxxxiii, 451.

On testing zymins from two different yeasts (Table III) it was found that removing the coenzyme very greatly decreased the rate of inorganic phosphate formation. The differences assume greater significance when it is remembered that in the unwashed material there was probably considerable simultaneous disappearance of inorganic phosphate due to esterification, a condition that did not obtain with the washed zymmin.

That the phenomenon was really one of complement enzyme action was shown by an experiment in which hexose diphosphate was acted upon by washed zymmin alone, by a hot water extract of fresh yeast, and by both together. It was found (Table IV) that while each portion separately was inactive, the mixture of the two rapidly dephosphorylated the diphosphate.

The effect of cozymase concentration was determined in a similar experiment in which it was found (Table V) that the rate of dephosphorylation increased regularly with increasing amount of coenzyme, although the maximum amount used was too small to establish the upper limit of the effect.

As such large differences were found in the rate of hydrolysis of the hexose diphosphate, the experiments were extended to the Robison and Neuberg esters and here again (Table VI) removal of the coenzyme very greatly reduced the phosphatase action.

These results in the presence of arsenate were direct and required the introduction of no assumptions. However, when the experiments were repeated, with the exception that the arsenate was omitted, it was found that the dephosphorylation was obscured by the esterification which accompanied fermentation. It was therefore assumed that the Harden and Young¹¹ equation



represents the course of fermentation, and that 1 mol of phosphate is esterified for each mol of carbon dioxide produced. This is probably a minimum figure as some esterification apparently occurs without carbon dioxide production, but serves as a first approximation. The increase in inorganic phosphate, and the amount of carbon dioxide produced, were therefore determined on separate portions of the same mixture and the sum of the two was

taken as being the amount of dephosphorylation. In other words, if expressed in mols:

Since PO_4 appearing = PO_4 formed by hydrolysis - PO_4 esterified
 and if PO_4 esterified $\geq \text{CO}_2$ produced
 then PO_4 formed by hydrolysis $\geq \text{PO}_4$ appearing + CO_2 produced.

It was found by experiment (Table VII) that the hydrolysis as thus calculated for the unwashed zymin was much greater than the measured hydrolysis produced by washed zymin. In the latter case there was of course no carbon dioxide production and therefore

TABLE I.
Hydrolysis of Diphosphate in 1 Hour (mM per Liter).

Zymin.	Lager.		Ale.		Brewery.	
	Control.	Arsenate.	Control.	Arsenate.	Control.	Arsenate.
Unwashed.....	0.3	>19.0	4.4	>15.8	6.2	>76.1
Washed.....	1.1	1.3	0.0	1.1	-2.0	-2.2

TABLE II.
Hydrolysis in 1 Hour (mM per Liter).

Zymin.	Ester.					
	Diphosphate.		Robison.		Neuberg.	
	Control.	Arsenate.	Control.	Arsenate.	Control.	Arsenate.
Unwashed.....	-6.2	>76.1	24.0	>30.5	26.0	>17.1
Washed.....	-2.0	-2.2	9.6	3.5	5.0	2.5

no correction for reesterification was necessary. It was interesting to learn that though the zymins had been very well washed, there was often some dephosphorylation and that its magnitude varied with the yeast employed. The lager zymin in particular showed a high residual rate and it was perhaps with some similar yeast that Harden and Young¹⁰ observed dephosphorylation after washing out the cozymase.

Results like those with the diphosphate were obtained on substituting the Robison and Neuberg esters. With these esters, however, the rate of residual hydrolysis after thorough washing, though much lower than that of the unwashed zymin, was still

very high (Table VIII). This residual rate was, moreover, not decreased by further washing.

In order to render the comparison of the various results somewhat easier, the amount of dephosphorylation in 60 minutes was calculated for each experiment and tabulated. In Table I are given the amounts found for the hexose diphosphate by use of three different zymins and in Table II the results with a single zymon on the diphosphate and on the Robison and Neuberg esters. In the arsenate experiments in which unwashed yeast was used the values are too low because of esterification and this is indicated by the symbol, >. In the other cases the values are correct as either there was no fermentation, or else it was already corrected for in the original calculations.

It will be observed that the washing (with a single exception) decreased the hydrolysis whether arsenate was present or absent, though the differences were greater in the former case. It will also be noted that even though the zymins had been very well washed, there was usually some residual hydrolysis. This raises the question as to whether a portion of the coenzyme cannot be removed by washing or, as alternative, whether there is a certain minimum rate which the coenzyme merely serves to increase. It is interesting that this residual hydrolysis varies with the yeast and with the substrate, being greatest for the lager zymon and for the Robison ester. In the case of the washed brewery yeast a slight synthesis may be seen. As a further point of interest it will be observed that while the arsenate accelerates the hydrolysis of the Robison and Neuberg esters in the case of the intact zymon, it produces an inhibition when the washed zymon is employed.

It would seem that all of these observations are of interest in connection with the as yet unsolved problems of the mechanism of arsenate and coenzyme action and that they deserve consideration on that account.

Cozymase and Live Yeast.

It is well known that the addition of toluene or chloroform to live yeast causes a decrease in the rate of carbon dioxide production and an accumulation of organic phosphates. It was in fact on this basis that Neuberg concluded that the organic phosphates were a result of abnormal conditions and did not represent a significant phase of zymase action.

However, in view of the above results a new interpretation appeared possible. If the effect of the toluene were to render the cell wall permeable to the cozymase (known to be dialyzable through ordinary membranes) then its concentration within the cell should decrease. The more complex zymase, being undialyzable, would remain within the cell and the resultant yeast would be quite similar to washed *zymin* in its behavior. The carbon dioxide production would be decreased by the lowered cozymase content, as would the hydrolytic activity toward phosphate esters. The esters, which in the normal yeast would be rapidly hydrolyzed and hence would not appear, now would continue to accumulate as long as the rate of phosphorylation was reasonably high.

This deduction was experimentally verified. On suspending fresh brewers' yeast in water and then centrifuging, it was found that the aqueous extract possessed little or no cozymase activity. On adding toluene to the extracting water the cell became permeable and considerable cozymase was found in the aqueous solution. An even larger amount was secured by a second toluene-water extraction (Table IX).

Another experiment along the same lines showed that of repeated short extractions of the yeast with water and toluene, the first contained but little cozymase, the second much more, the third almost as much as the second, and the fourth again very little.

To see how the purity of the cozymase as thus secured compared with that obtained by boiling water extracts, the solids were determined on the samples of the previous experiment and the A Co (maximum rate in cc. per hour per gm. of solid) calculated. For the boiling water extract the A Co was 40, for the second toluene-water extract 62, and for the third 107. The first and fourth extracts contained so little cozymase that the calculation was not justified (Table X).

The yield of coenzyme by the cold water-toluene method was also very good, the total yield of the second and third together exceeding that of the boiling water by 20 to 30 per cent.

Several conclusions result from these studies. A new function for cozymase has been established and an explanation of the peculiar effects of certain antiseptics on live yeast has been suggested. In addition a simple method of obtaining relatively pure cozymase in good yield has been discovered. The results obviously suggest

a number of related problems and an elaboration and extension of these studies is planned.

EXPERIMENTAL.

Methods.—The experimental technique and sources of material have been already described.¹² Carbon dioxide was determined by measuring the increase in pressure at constant volume in tubes that were kept at 25.0° and constantly shaken to liberate the gas. The change in inorganic phosphate was measured by the colorimetric method of Kuttner and Cohen¹⁶ which had been found¹² specific for inorganic phosphate.

Experiment I.—Zymins were prepared from lager and ale yeast¹⁷ and washed five and four times respectively by suspending in 15 volumes of water and centrifuging. The residue was diluted to a known volume and the equivalent of 0.4 gm. of original zymin was used. For parallel experiments 0.4 gm. of the unwashed zymins was also employed. To all four there were added 0.4 cc. of 0.04 molal sodium arsenate solution acidified to pH 6.4 and 1.0 cc. of 0.24 molal sodium hexose diphosphate similarly acidified. Each mixture was diluted to a total volume of 4 cc., giving an arsenate concentration of 0.004 mols per liter, hexose diphosphate 0.06 mols per liter, and zymin 10 per cent. Toluene was added, 1 drop per cc. The mixtures were kept in the thermostat at 25.0° and shaken to avoid settling; samples were taken after 10, 40, 75, and 130 minutes. The inorganic phosphate was determined after removal of the yeast and dilution. The measured concentrations of inorganic phosphate in the mixtures and the increase with time are given in Table III.

Experiment II.—Zymin from a bottom brewery yeast was washed three times as above.

Cozymase extract was prepared by adding 1000 cc. of boiling water to 200 gm. of fresh brewery yeast, stirring 10 minutes, cooling, centrifuging, filtering, and concentrating fivefold under reduced pressure.

Three mixtures were prepared, the total volume of each being 4

¹⁶ Kuttner, T., and Cohen, H. R., *J. Biol. Chem.*, 1927, lxxv, 517.

¹⁷ The lager and ale yeasts used in these experiments were presented to us by the Wallerstein Laboratories and the bottom yeast by the George Ehret Brewery. We are most grateful to them both for their kindness.

cc. They contained arsenate, 0.004 molal, and hexose diphosphate 0.06 molal, each at pH 6.4 and in addition: (a) washed zymin equivalent to 0.4 gm. of dry zymin; (b) 1.0 cc. of cozymase extract; (c) washed zymin equivalent to 0.4 gm. of dry zymin and 1.0 cc. of cozymase extract; toluene in each.

The measured inorganic phosphate concentrations and the calculated increases are given in Table IV.

TABLE III.
Inorganic Phosphate (mM per Liter).

Time.	Concentration (measured).				Increase (calculated).			
	Lager.		Ale.		Lager.		Ale.	
	Un-washed.	Washed.	Un-washed.	Washed.	Un-washed.	Washed.	Un-washed.	Washed.
<i>min.</i>								
10	17.3	4.5	14.7	4.6	0	0	0	0
40	27.7	5.5	23.9	5.2	10.4	1.0	9.2	0.6
75	42.7	6.1	35.4	6.0	25.4	1.6	20.7	1.4
130	57.9	7.3	49.3		40.6	1.8	34.6	

TABLE IV.
Inorganic Phosphate (mM per Liter).

Time.	Concentration (measured).			Increase (calculated).		
	Washed zymin.	Cozymase.	Washed zymin + cozymase.	Washed zymin.	Cozymase.	Washed zymin + cozymase.
<i>min.</i>						
10	12.0	24.4	26.4	0	0	0
40	10.7	24.2	38.4	-1.3	-0.2	12.0
75	9.5	24.4	52.2	-2.5	0.0	25.8
110	8.8	24.1	62.5	-3.2	-0.3	36.1

Experiment III.—Washed zymin and cozymase solution were prepared as in Experiment II.

Mixtures were prepared containing 0.4 gm. of zymin and 0.0, 0.02, 0.1, 0.5, and 1.5 cc. of cozymase extract in a volume of 4 cc. The mixtures were also made 0.004 molal with respect to arsenate and 0.06 molal in hexose diphosphate, pH 6.4. A sixth mixture was prepared, similar to the above, only containing no cozymase

extract and with unwashed zymin instead of washed. To all mixtures toluene was added. The inorganic phosphate concentrations and the increases are given in Table V.

Experiment IV.—This experiment was similar to Experiment I only with use of brewery yeast zymin, and Robison and Neuberg esters in place of the diphosphate.

The fermentation mixtures contained zymin 10 per cent, arsenate 0.004 molal, esters 0.06 molal; toluene was added. The

TABLE V.
Increase in Inorganic Phosphate (mM per Liter).

Time.	Unwashed zymin.	Washed zymin + yeast extract, cc. extract per 10 cc.				
		0.0	0.05	0.25	1.25	3.75
<i>min.</i>						
10	0	0	0	0	0	0
40	52.9	-1.6	-1.5	1.0	8.4	22.8
70	87.7	-2.6	-2.2	3.7	19.3	37.9

TABLE VI.
Inorganic Phosphate (mM per Liter).

Time.	Concentration (measured).				Increase (calculated).			
	Robison.		Neuberg.		Robison.		Neuberg.	
	Un- washed.	Washed.	Un- washed.	Washed.	Un- washed.	Washed.	Un- washed.	Washed.
<i>min.</i>								
10	23.6	7.8	24.1	6.1	0	0	0	0
55	42.0	10.9	40.0	8.3	17.4	3.1	15.9	2.2
120		15.5		11.7		7.7		5.6

inorganic phosphate concentrations and the calculated increases are given in Table VI.

Experiment V.—This experiment was similar to Experiment I only with use of three zymins, lager, ale, and brewery. The mixture contained zymin 10 per cent and hexose diphosphate 0.06 molal; toluene was added. Both CO₂ and phosphate changes were determined on samples of the unwashed mixtures and the sum of the two was taken as a measure of the dephosphorylation (Table VII).

Experiment VI.—This experiment was similar to Experiment IV only with no arsenate. The mixture contained zymyn 10 per cent and esters 0.06 molal; toluene was added. Both CO_2 and phosphate changes were measured on the unwashed mixtures and the sum of the two was used as a measure of the dephosphorylation (Table VIII).

TABLE VII.
Hydrolysis in Absence of Arsenate.

Time.	Ale yeast.				Lager yeast.				Brewery yeast.			
	Unwashed.			Washed.	Unwashed.			Washed.	Unwashed.			Washed.
	PO_4	CO_2	Total.		PO_4	CO_2	Total.		PO_4	CO_2	Total.	
<i>min.</i>												
10	0	0	0	0	0	0	0	0	0	0	0	0
40	-4.4	5.8	1.4		-2.4	2.1	-0.3	0.8	-0.1	3.2	3.3	-1.3
75	-5.0	11.3	6.3	0.0	-5.2	5.9	0.7	1.3	1.5	6.8	8.3	-2.5
130	-5.3	19.8	14.8	0.8	-6.2	11.7	5.5	2.3	8.8	12.2	21.0	-3.2

TABLE VIII.
Hydrolysis in Absence of Arsenate.

Time.	Robison.				Neuberg.			
	Unwashed.			Washed.	Unwashed.			Washed.
	PO_4	CO_2	Total.		PO_4	CO_2	Total.	
<i>min.</i>								
10	0	0	0	0	0	0	0	0
55	4.3	19.0	23.3	9.0	6.6	18.5	25.1	4.5
120	3.2	29.2	32.4	15.6	6.8	29.4	36.2	11.4

Experiment VII.—200 gm. of fresh brewery yeast were suspended in 500 cc. of water and stirred with a motor for 20 minutes. The mixture was centrifuged, the supernatant liquid saved, and the solid resuspended in the same volume of water. After 20 minutes, it was centrifuged as before and resuspended, only this time 25 cc. of toluene were added to the mixture. The process was repeated once more, toluene being again added.

The solutions were all concentrated five times under reduced

pressure and tested for cozymase content by adding them to washed zymin. The fermentation mixtures contained 10 per cent zymin (dry weight), 10 per cent glucose, 0.033 molal phosphate buffer (pH 6.4), 0.005 molal hexose diphosphate (to remove induction), and 2.5 cc. of cozymase per 10 cc. Toluene was added in each case and the fermentation was measured at 25.0°. The data were plotted and from the graph the maximum rate and

TABLE IX.
Extraction of Cozymase from Live Yeast.

		Maximum rate.	Total in 3 hrs.
		cc. per hr.	cc.
First extract.	No toluene.....		0.2
Second	" " "		0.1
Third	" Toluene.....	1.3	2.5
Fourth	" "	2.8	4.5

TABLE X.
Extraction of Cozymase from Live Yeast.

Extract.	Total in 2½ hrs.	Maximum rate.	Solids.	A Co.
	cc. per cc. extract	cc. per hr. per cc. extract	gm. per cc.	
Toluene 1.....	0.7	0.4		
" 2.....	3.6	2.1	0.034	62
" 3.....	2.6	1.6	0.015	107
" 4.....	0.6	0.4		
Boiling H ₂ O.....	4.6	3.1	0.077	40

the total CO₂ in 3 hours were obtained. These are given in Table IX.

Experiment VIII.—This experiment was similar to Experiment VII except that toluene was added in making all four extracts. All were concentrated fivefold under reduced pressure.

Another extract was prepared from fresh yeast with boiling water as in Experiment II but was concentrated tenfold to make the ratio, grams yeast to cc. of extract, the same as for the toluene-water extracts. 2.5 cc. of each were used in the cozymase assay which was done as in Experiment VII.

The solid content of each extract was determined by drying in air, and then over P_2O_5 in high vacuum to constant weight.

The total CO_2 for $2\frac{1}{2}$ hours, the maximum rate in cc. per hour for 1 cc. of extract, the solid content, and the calculated A Co are given in Table X.

SUMMARY.

1. Cozymase was found to be involved in the action of the phosphatase of yeast. Removing the coenzyme from zymine by washing decreased the hydrolysis of hexose diphosphate both in presence and absence of arsenate. The differences in the presence of arsenate were much greater than in its absence.

Similar results were obtained with zymines from three different yeasts. The hydrolysis was not completely stopped no matter how thorough the washing, and the rate of residual hydrolysis varied considerably with the yeast employed.

Both the Robison and Neuberg esters behaved like the diphosphate but the rate of residual hydrolysis after washing was much higher in their case.

2. The effect was one of complement enzyme action; neither washed zymine nor cozymase produced significant dephosphorylation of hexose diphosphate while the mixture of the two was active in this respect.

The rate of hydrolysis by washed zymine increased with increasing amount of cozymase within the limits of concentration employed.

3. Cozymase was not extracted from live yeast with water, but in the presence of toluene, large amounts were extracted.

The yield and purity of the coenzyme obtained by this method exceeded those of boiling water extracts of the same yeast.

BLOOD SUGAR DETERMINATION AND SEPARATION OF SUGARS WITH LIVE YEAST.

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It is of the greatest importance for both clinical and experimental purposes, to be able to analyze mixtures of different sugars, and in particular to determine glucose in the presence of other sugars. So many procedures have been proposed for this purpose that it is impossible to discuss them, but it may be said that in general they fall into two classes: chemical methods depending upon different rates of reaction of the various sugars under particular empirical conditions, and fermentation methods depending upon selective action of living organisms. The first group suffers from the fact that such chemical differentiation is in general not delicate enough to distinguish between closely related substances, while the second type is usually much too slow or too tedious to be of general utility.

Somogyi^{1,2} recently observed that bakers' yeast removes glucose quantitatively from dilute solution in a very short time and that this action is limited to the fermentable sugars. As we had need of a method of rapid differential analysis of sugars in mixtures, and as Somogyi gave no data except on glucose, we decided to study the possibilities of the method. Our results confirm Somogyi's observations and help to indicate that this method of analysis should prove very valuable.

A technique was developed which permitted the use of as little as 0.3 cc. of blood or solution—0.1 cc. for the determination of total reducing substances and 0.2 cc. for the determination of the non-adsorbed fraction. The Hagedorn-Jensen method was employed throughout for the reduction determinations.

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¹ Somogyi, M., *Proc. Soc. Exp. Biol. and Med.*, 1926-27, xxiv, 320.

² Somogyi, M., *J. Biol. Chem.*, 1927, lxxv, 33.

It was found that under the conditions employed, lactose, galactose, maltose, xylose, arabinose, ribose, and dihydroxyacetone remained quantitatively in the filtrate after yeast treatment. The experiments were performed on pure aqueous solutions at concentrations corresponding to blood sugar levels of 100 and 200 mg. per 100 cc. Under the same conditions, mannose, fructose, and sucrose were adsorbed to the extent of 60 to 80 per cent but the results were variable and depended upon the time of adsorption, the concentration of the sugar, and the state of the yeast.

Glucose, however, was quantitatively adsorbed from aqueous solution at concentrations corresponding to 500 mg. per 100 cc. and in the absence of added protein. This differs apparently from Somogyi's findings, as he seems to think that the protein is necessary. We did, however, use tungstic acid in all cases and this perhaps coagulated the yeast just as though foreign protein were present.

By using mixtures of glucose with several of the unadsorbed sugars, it was found that the action of the yeast was selective and that no interference took place. The glucose was again quantitatively adsorbed and the reduction value obtained was exactly that of the non-adsorbable sugar which was introduced.

In order to make sure that the method was applicable to blood as well as to aqueous solutions, fresh blood was added to known amounts of the non-adsorbed sugars and the reduction was determined before and after treatment with yeast. With one exception the results were the same as before; *i.e.*, no interference took place and the substances were quantitatively recovered in the filtrate. In the case of maltose, however, a very appreciable loss occurred, amounting to about 40 per cent. Whether this was due to hydrolysis or adsorption by the blood, or to adsorption by the yeast induced by the blood, was not determined.

In employing the method it was necessary to apply corrections for the reduction contributed by the yeast, but such corrections were not large and were extremely constant.

EXPERIMENTAL.

Yeast.—The yeast employed was Fleischmann's bakery yeast,³ supplied in pound or half pound packages. It was prepared for

³ We wish to thank the Fleischmann Yeast Company for their kindness in giving us the yeast for these experiments.

use as indicated by Somogyi—washed thoroughly by being suspended in water and centrifuged. This operation was repeated until the supernatant liquid had not the slightest turbidity—usually six or seven washings sufficed. The moist, well centrifuged material was then weighed out and suspended in 3 times its own weight of distilled water.

When fresh, the yeast suspension contributed no reducing substance but after a few days, some reduction was observed. We therefore made a practice of determining the blank each day and correcting all observed values by the amount found.

The yeast suspension, kept in the ice box, could be used for 2 weeks or longer, particularly if, as Somogyi suggests, the water was centrifuged off and replaced.

Sugars.—The pentoses were prepared in this laboratory and were quite pure. They were used without further treatment.

The fructose employed was levulose, Pure, Eimer and Amend. It was four times recrystallized from aqueous alcohol to give a beautiful, colorless, crystalline product.

Mannose and galactose were pure preparations made in this laboratory. Both were twice recrystallized from aqueous alcohol for further purification.

Lactose was a Kahlbaum preparation and was four times recrystallized from water before being used.

Maltose, sucrose, and glucose were high grade commercial preparations. They were used without further treatment.

Dihydroxyacetone, monomeric, was prepared by distilling oxantin⁴ (Metz) under greatly reduced pressure. The distilled solid was thoroughly washed with a mixture of equal volumes of absolute alcohol and absolute ether, dried in high vacuum over phosphorus pentoxide, and kept in the ice box. Only freshly made solutions were employed.

In case of any doubt, the purity of the sugars was confirmed by measurements of the optical rotation.

Stock solutions, approximately 2 per cent, were prepared from all the sugars and diluted as desired. No great accuracy was attempted in making up the solutions but the determinations and standardizations were done on the same sample, which cancels out errors of dilution.

⁴We are indebted to the H. A. Metz Laboratories, Inc., for the oxantin which was used in this work.

Reduction Method.—The Hagedorn-Jensen⁵ method was used for all the determinations. It was found from preliminary experiments that after precipitation of the yeast with tungstic acid and centrifugation, the zinc hydroxide treatment used in the Hagedorn-Jensen method could be omitted as the results were identical within the limits of experimental error. This was the only modification which was introduced.

Technique.—To 2.5 cc. of a solution of the desired sugar there was added 1.0 cc. of the 25 per cent (wet weight) yeast suspension. The contents were mixed by swirling and tapping against the hand and after 2 minutes there was added 0.5 cc. of tungstic acid solution (prepared freshly each day by mixing equal volumes of 10 per cent sodium tungstate and $\frac{2}{3}$ N sulfuric acid). The test-tube was then covered with the thumb and mixed by quickly inverting it a few times. Shaking was avoided as a froth was formed which would not centrifuge down.

After 3 minutes the tubes were placed in wooden adapters and centrifuged at high speed for 5 minutes. The cake was compact and firm, and there were about 3 cc. of clear solution. Either 2.0 or 1.0 cc. of the supernatant liquid (depending upon the amount of reducing substance present) was removed with a pipette and transferred to a 25 × 150 mm. Pyrex test-tube for the reduction determination. Before addition of the potassium ferricyanide the sample was diluted to approximately 9 cc. to conform to the usual procedure.

Where blood was used, 0.2 cc. was collected in a pipette and transferred to 2.3 cc. of distilled water, the pipettes being rinsed by sucking up the solution once or twice. This mixture was used to replace the 2.5 cc. of sugar solution in the above procedure.

Test-tubes were used for the precipitation as they were much easier to clean, and being of small diameter, produced a higher liquid column than the ordinary 15 cc. centrifuge tube.

Adsorptions.—The amounts of sugar actually employed in a determination were 0.2 or 0.4 mg. which corresponds to 100 and 200 mg. per 100 cc. if a 0.2 cc. sample is used. No great accuracy was attempted in preparing the stock solutions as the determinations and standardizations were done on the same sample of

⁵ Hagedorn, H. C., and Jensen, B. N., *Biochem. Z.*, 1923, cxxxv, 46.

solution. Since only the ratio of the two was used, no error was introduced.

The yeast contributed a certain reduction value, particularly after it has stood for several days, and blank runs were therefore made each day and the corresponding correction was applied in

TABLE I.
Percentage of Sugar Not Adsorbed by Yeast.

Substance.	Amount of sugar in sample used.	
	0.2 mg.	0.4 mg.
	<i>per cent</i>	<i>per cent</i>
Dihydroxyacetone.....	103	101
Xylose.....	100	99.5
Ribose.....	102	99.5
Arabinose.....	100.5	99
Galactose.....	101	97
Maltose.....	103	97
Fructose.....	40	42
Mannose.....	26	34
Sucrose.....	59	63

TABLE II.
Effect of Time on Adsorption.

Time.	Per cent adsorbed.	
	Mannose.	Fructose.
<i>min.</i>		
3	39	36
5	51	49
7	63	64
11	76	79
18	87	91
33	86	95

making the calculations. The results in Table I, for two concentrations of each of the sugars, are based upon the average of four determinations which did not differ by more than ± 2 per cent.

To study the time effect for those substances which were partially adsorbed, mannose and fructose were subjected to the

action of the yeast for varying periods. The results are given in Table II. The effect of concentration was also determined for these two sugars and is given in Table III. Two yeasts were used; one was freshly prepared, while the other had been made up about 3 weeks previously.

A further experiment was done to see whether the action of the yeast remained selective when mixtures were used. Glucose,

TABLE III.
Effect of Sugar Concentration on Adsorption.

Mg. in sample.	Per cent absorbed.			
	New yeast.		Old yeast.	
	Mannose.	Fructose.	Mannose.	Fructose.
0.1	77	63		80
0.2	74	60	79	75
0.4	69	58	60	84
0.7	69	56	56	
1.0	69	58	59	86
2.0	66	55		72

TABLE IV.
Adsorption of Glucose from Mixture.
1.0 mg. glucose + 0.20 mg. sugar.

Sugar.	Found (average).	
	mg.	per cent
Galactose.....	0.208	104
Lactose.....	0.203	101.5
Maltose.....	0.195	97.5
Dihydroxyacetone.....	0.201	100.5
Xylose.....	0.200	100

1.0 mg., was mixed with 0.2 mg. of certain of the non-adsorbed sugars and the mixture was subjected to the action of the yeast. The reduction value found for the filtrate was divided by that of the non-adsorbable sugar originally introduced. Had the adsorption of the glucose been interfered with by the other sugars present, then the results would have been appreciably higher than 100 per cent. Actually, this was not the case as is seen in Table IV.

The effect of blood on the adsorption of glucose had already been studied by Somogyi.² It was therefore only necessary for us to see if the addition of blood modified the results with the non-adsorbed substances. For this purpose 0.2 cc. of fresh blood was added to a known amount of the desired sugars in 2.3 cc. volume and the mixture was treated as usual. A blank was run with blood without additional sugar and all values were decreased by the amount found for this blank. It was again found that dihydroxyacetone, xylose, ribose, arabinose, galactose, and lactose remained quantitatively in solution after the yeast treatment. With maltose, however, only about 60 per cent was recovered in the filtrate giving an adsorption of 40 per cent by the yeast-blood mixture.

SUMMARY.

1. The observations of Somogyi on the adsorption of glucose and non-adsorption of unfermentable sugars have been confirmed.

2. A technique is described employing 0.3 cc. of blood or aqueous solution, 0.2 cc. for the adsorption and 0.1 cc. for total reduction.

3. Dihydroxyacetone, xylose, ribose, arabinose, galactose, lactose, and maltose in aqueous solution were recovered quantitatively in the filtrate after yeast treatment.

Fructose, mannose, and sucrose were partially adsorbed. The extent of the adsorption varied with the time of adsorption, concentration of the sugar, and state of the yeast.

Glucose was quantitatively adsorbed at concentrations corresponding to blood sugar levels of 500 mg. per 100 cc.

4. The action of the yeast was selective, only glucose was removed when mixed with sugars of the non-adsorbed group.

5. The results were the same whether blood or aqueous solutions were employed except in the case of maltose. This sugar was adsorbed to the extent of about 40 per cent in the presence of blood.

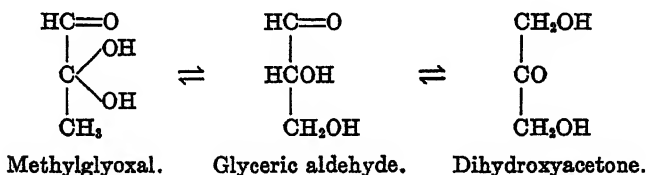
DIHYDROXYACETONE AND INSULIN HYPOGLYCEMIA.

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It is generally assumed that in the organism the combustion of glucose proceeds in several phases and that the first phase consists of the dissociation of glucose into two trioses. Of these the simplest form is glyceric aldehyde which may tautomerize into methylglyoxal or dihydroxyacetone.



In recent years claims have been made that of the three trioses, dihydroxyacetone is the most probable intermediate product in the process of sugar combustion. It has been shown by experiments on animals with pancreatic and phlorhizin diabetes that it is transformed in the organism into glucose.¹ The same conclusions were drawn from perfusion experiments. From respiration experiments, the conclusion was reached that dihydroxyacetone is burned in the organism with greater velocity than glucose.^{2,3}

These experimental conclusions seemed to have justified the suggestion of Emil Fischer that dihydroxyacetone could serve as a substitute for glucose, and the high velocity of its combustion suggested its application in diabetes. It must be added, however,

¹ Campbell, W. R., and Markowitz, J., *Am. J. Physiol.*, 1927, lxxx, 561.

² Cathcart, E. P., and Markowitz, J., *J. Physiol.*, 1927, lxiii, 309.

³ Himwich, H. E., Rose, M. I., and Maler, M. R., *Proc. Soc. Exp. Biol. and Med.*, 1926-27, xxiv, 238.

that there is no unanimity of opinion as to the fate of dihydroxyacetone in the organism and nearly every favorable claim for its usefulness has been controverted. In recent years a new method was developed for testing possible intermediate products of sugar metabolism. The method was based on the idea that if a substance is readily converted into glucose or if it is an intermediary product of glucose metabolism, it should be able to replace glucose in arresting hypoglycemic convulsions. Dihydroxyacetone has been tested by this method and again the results of the experiments are conflicting. Kermack, Lambie, and Slater⁴ and Campbell and Hepburn⁵ report favorable results, whereas Markowitz and Campbell⁶ deny the claim that dihydroxyacetone is capable of abating hypoglycemic convulsions in animals deprived of the liver.

The controversy between the different writers could be explained in part, it seemed to us, by two factors. First, by the fact that the product oxantin (Höchst), which has been generally employed, was shown to contain a considerable quantity of polymerization products, particularly when the oxantin was not absolutely fresh. Second, some workers did not differentiate sufficiently between temporary and permanent cure of the hypoglycemic symptoms.

H. O. L. Fischer⁷ has recently prepared crystalline monomolecular dihydroxyacetone by distillation under reduced pressure of the polymerized forms. Thus the pure monomolecular form of dihydroxyacetone was readily accessible.

Recently Somogyi⁸ made the very important observation that yeast possesses specific adsorbing power for the fermentable sugars. These statements were confirmed by Raymond and Blanco and will be published in a separate article. On the basis of these observations a method was developed for the quantitative estimation of dihydroxyacetone in the blood after subcutaneous or intravenous injection. Thus the effect of dihydroxyacetone could be studied as a function of its concentration in the blood.

⁴ Kermack, W. O., Lambie, C. G., and Slater, R. H., *Biochem. J.*, 1926, **xx**, 486.

⁵ Campbell, W. R., and Hepburn, J., *J. Biol. Chem.*, 1926, **lxviii**, 575.

⁶ Markowitz, J., and Campbell, W. R., *Am. J. Physiol.*, 1927, **lxxx**, 548.

⁷ Fischer, H. O. L., and Mildbrand, H., *Ber. chem. Ges.*, 1924, **lvii B**, 707.

⁸ Somogyi, M., *Proc. Soc. Exp. Biol. and Med.*, 1926, **xxiv**, 320; *J. Biol. Chem.*, 1927, **lxxv**, 33.

TABLE I.

Time.	Remarks.	Time.	Remarks.
Experiment 1. Rabbit; weight 2200 gm.			
10.00 a.m.	15 units insulin.	<i>p.m.</i>	
<i>p.m.</i>		3.28	Blood glucose, 36 mg. per 100 cc. blood.
1.30	Convulsions.		DHA, 26 mg. per 100 cc. blood.
1.35	Recovered.		
2.30	Convulsions.		
2.32	Recovered.	3.35	In coma.
2.40	Convulsions.	3.37	Convulsions.
2.43	Recovered.	3.45	Recovery.
2.47	Convulsions.	3.48	In coma.
2.50	Recovered.	3.52	Blood glucose, 23 mg. per 100 cc. blood.
2.55	Convulsions.		DHA, 15 mg. per 100 cc. blood.
3.00	Blood glucose, 21 mg. per 100 cc. blood.		
3.05	0.5 gm. DHA,* sub- cutaneously.	3.55	0.75 gm. DHA.
		4.05	Recovered.
3.14	No recovery.	4.10	Blood glucose, 32 mg. per 100 cc. blood.
3.16	0.75 gm. DHA, venous.		DHA, 37 mg. per 100 cc. blood.
3.20	Recovered.		
		4.30	Convulsions.
Experiment 2. Rabbit; weight 2000 gm.			
11.00 a.m.	15 units insulin.	<i>p.m.</i>	
<i>p.m.</i>		3.30	In coma.
1.15	Convulsions.	3.45	Blood glucose, 18 mg. per 100 cc. blood.
1.30	Recovered.		DHA, 19 mg. per 100 cc. blood.
2.00	Convulsions.		
2.20	Blood glucose, 29 mg. per 100 cc. blood.	3.50	0.75 gm. DHA, venous.
2.25	0.5 gm. DHA, venous.	3.52	Recovered.
2.27	Recovered.	3.54	In coma.
2.28	Convulsions.	4.05	0.75 gm. DHA, venous.
2.35	1 gm. DHA, venous.	4.25	Still in coma.
2.40	Recovered.	4.30	Blood glucose, 23 mg. per 100 cc. blood.
2.50	Blood glucose, 37 mg. per 100 cc. blood.		DHA, 22 mg. per 100 cc. blood.
	DHA, 87 mg. per 100 cc. blood.		

* DHA is used as an abbreviation for dihydroxyacetone.

TABLE I—Continued.

Time.	Remarks.	Time.	Remarks.
Experiment 3. Rabbit; weight 1600 gm.			
9.45 a.m.	20 units insulin.	<i>p.m.</i>	
<i>p.m.</i>		1.55	Convulsions.
1.15	Convulsions.	2.00	0.3 gm. DHA, venous.
1.20	0.25 gm. DHA, subcutaneously. No recovery.	2.10	Convulsions.
		2.15	0.25 gm. DHA, venous.
1.25	0.5 gm. DHA, venous.	2.20	Convulsions.
1.35	No convulsions but in coma.	2.22	0.25 gm. DHA, venous.
		3.15	Coma and convulsions.
Experiment 4. Rabbit; weight 1800 gm.			
10.05 a.m.	20 units insulin.	<i>p.m.</i>	
<i>p.m.</i>		1.55	0.30 gm. DHA, venous.
1.15	Coma and convulsions.		0.60 " " subcutaneously.
1.30	0.25 gm. DHA, venous.		
1.36	0.25 " " "	2.05	No recovery.†
1.45	0.40 " " "		
	No recovery.		
Experiment 5. Rabbit; weight 2400 gm.			
10.00 a.m.	20 units insulin.	<i>p.m.</i>	
<i>p.m.</i>		5.48	Recovery.
4.48	Coma and convulsions.	6.10	Coma.
5.02	0.5 gm. DHA, subcutaneously.	6.14	Convulsions.
		6.16	0.5 gm. DHA, subcutaneously.
5.05	Recovery.		
5.20	Coma.	6.20	Recovery.
5.35	Convulsions.	6.35	Coma and convulsions.†
5.44	0.5 gm. DHA, subcutaneously.		

† Recovery in 12 min. with 0.85 gm. of glucose subcutaneously.

‡ Complete recovery with 1 gm. of glucose subcutaneously.

TABLE I—*Continued.*

Time.	Remarks.	Time.	Remarks.
Experiment 5 a. Rabbit; weight 2400 gm.			
9.45 a.m.	20 units insulin.	<i>p.m.</i>	
<i>p.m.</i>		5.30	Convulsions.
4.00	Coma.	5.45	0.20 gm. DHA, venous.
4.15	Convulsions.	6.00	0.20 " " "
4.25	0.30 gm. DHA, venous.	6.15	0.20 " " "
4.28	Recovery.	6.25	0.20 " " "
5.05	Convulsions.	6.35	No recovery.
5.10	1 mg. ergotamine, venous.	6.35	0.75 gm. glucose, subcutaneously; recovery.
5.15	0.30 gm. DHA, venous.		
5.25	Convulsions.		
Experiment 6. Rabbit; weight 1600 gm.			
10.00 a.m.	25 units insulin.	<i>p.m.</i>	
<i>p.m.</i>		2.03	Recovery.
1.30	Coma.	2.06	Convulsions.
1.45	Convulsions.	2.09	0.5 gm. DHA, venous.
1.53	0.5 gm. DHA, subcutaneously.	2.10	Recovery.
1.55	Recovery.	2.14	Coma and convulsions with alternate moments of recovery.
1.57	Convulsions and coma.	2.35	
2.02	0.5 gm. DHA, venous.	3.36	Deep coma. §
Experiment 7. Rabbit; weight 2050 gm.			
10.50 a.m.	30 units insulin.	<i>p.m.</i>	
<i>p.m.</i>		3.38	Blood glucose, 110 mg. per 100 cc. blood; no DHA.
2.00	Convulsions.		
2.30	Blood glucose, 9 mg. per 100 cc. blood.	4.15	Blood glucose, 61 mg. per 100 cc. blood; no DHA.
2.42	2 gm. DHA, subcutaneously.		
2.48	Recovery.	4.55	Blood glucose, 69 mg. per 100 cc. blood.
3.13	Blood glucose, 33 mg. per 100 cc. blood; no DHA.	6.30	Coma, convulsions.

§ Complete recovery with 2 gm. of glucose.

TABLE I—*Concluded.*

Time.	Remarks.	Time.	Remarks.
Experiment 8. Rabbit; weight 2100 gm.			
10.30 a.m.	30 units insulin.	<i>p.m.</i>	
<i>p.m.</i>		3.50	In coma.
3.10	Convulsions.	4.10	Convulsions.
3.15	2 gm. DHA, subcutaneously.	4.15	Blood glucose, 5 mg. per 100 cc. blood.
3.25	Recovery.		DHA, 14 mg. per 100 cc. blood.
3.30	Blood glucose, 24 mg. per 100 cc. blood. DHA, 8 mg. per 100 cc. blood.		
Experiment 9. Rabbit; weight 1650 gm.			
10.40 a.m.	40 units insulin.	<i>p.m.</i>	
<i>p.m.</i>		4.45	Began to recover.
2.30	Convulsions.	4.50	Blood glucose, 22 mg. per 100 cc. blood.
2.43	2 gm. DHA, subcutaneously.		DHA, 94 mg. per 100 cc. blood.
3.20	No recovery.		
3.25	2 gm. DHA, subcutaneously.	5.50	Blood glucose, 36 mg. per 100 cc. blood.
3.50	Blood glucose, 9 mg. per 100 cc. blood. DHA, 184 mg. per 100 cc. blood.		DHA, 15 mg. per 100 cc. blood.
		6.20	Began to fall into coma.
		7.00	Exitus in coma.

The present investigation deals with the effect of pure monomeric dihydroxyacetone on the course of hypoglycemia produced by administration of insulin. The mode of preparation of the animals will be given in the experimental part. The results are summarized in Table I and in Figs. 1 to 5.

In a general way it was observed that both subcutaneous and intravenous administrations of dihydroxyacetone were often followed by temporary recovery from the symptoms of hypoglycemia. The recovery was temporary after large as well as after repeated small fractional doses of dihydroxyacetone.

In all cases of temporary recovery after dihydroxyacetone

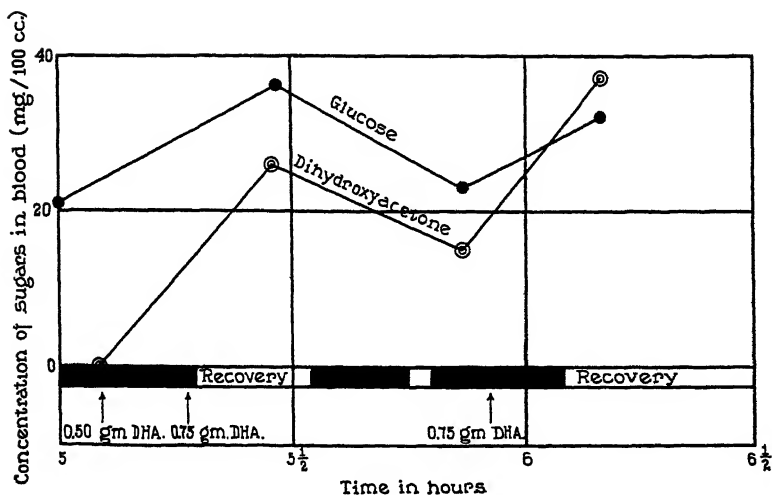


FIG. 1. Effect of dihydroxyacetone on insulin convulsions, and concentration of dihydroxyacetone and glucose in blood. Experiment 1.

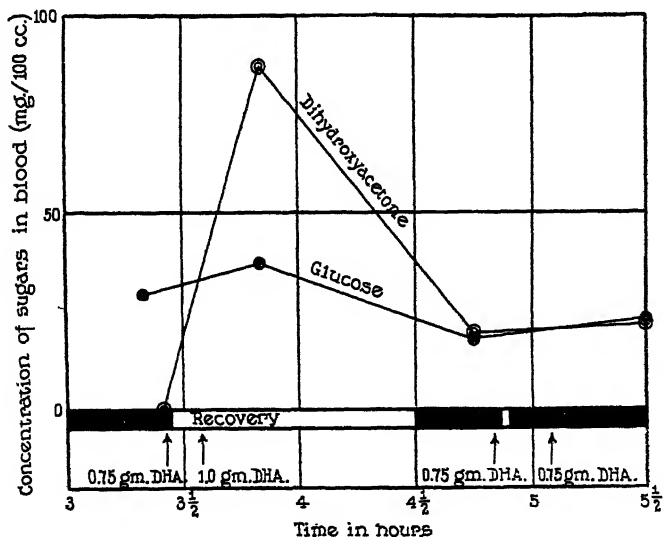


FIG. 2. Effect of dihydroxyacetone on insulin convulsions, and concentration of dihydroxyacetone and glucose in blood. Experiment 2.

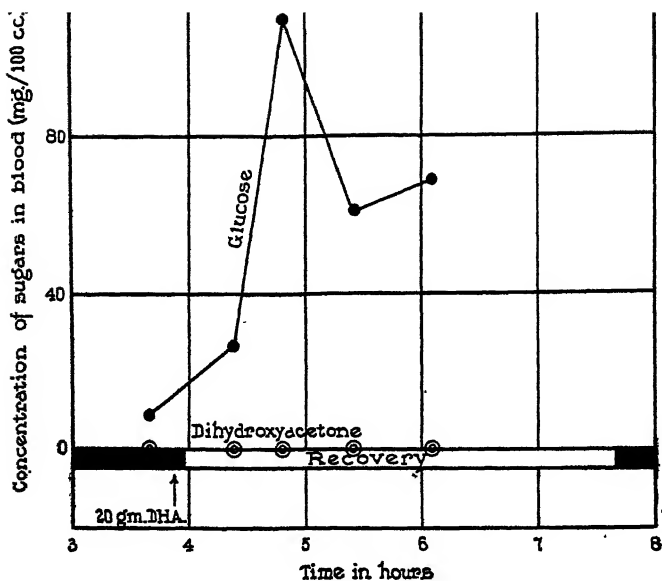


FIG. 3. Effect of dihydroxyacetone on insulin convulsions, and concentration of dihydroxyacetone and glucose in blood. Experiment 7.

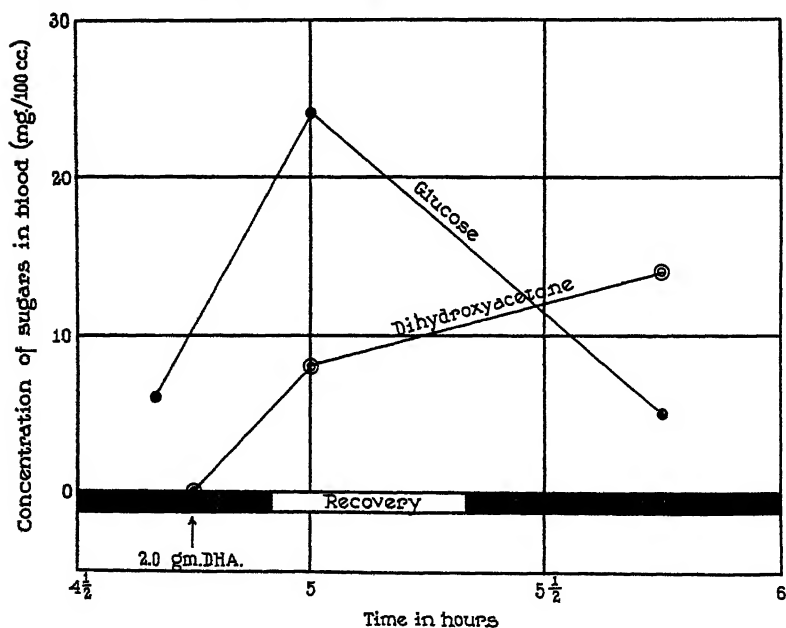


FIG. 4. Effect of dihydroxyacetone on insulin convulsions, and concentration of dihydroxyacetone and glucose in blood. Experiment 8.

administration, there was noted a rise in the concentration of glucose in the blood. In one case no dihydroxyacetone was found in the blood (Fig. 3). In other cases simultaneously with the increase in the blood sugar there was noted the presence of dihydroxyacetone (Figs. 4 and 5).

In the cases treated with repeated small doses of dihydroxyacetone the earlier injections which were followed by temporary recovery were followed also by increase in the blood glucose; later injections which failed to bring about recovery did not raise the

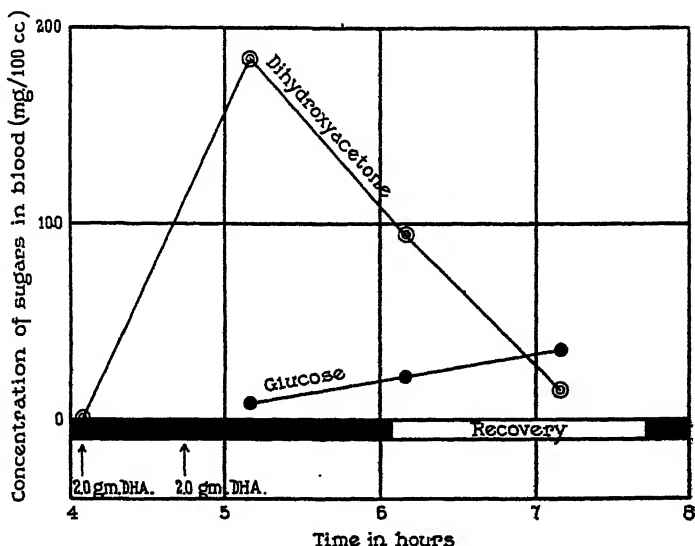


FIG. 5. Effect of dihydroxyacetone on insulin convulsions, and concentration of dihydroxyacetone and glucose in blood. Experiment 9.

concentration of the blood sugar (Figs. 1 and 2). In all these cases the injections of dihydroxyacetone were followed by the appearance of the substance in the blood.

The present experiments give no definite answer to the question as to the source of the surplus sugar appearing in the blood after the administration of dihydroxyacetone. It is doubtful that it is formed by condensation of dihydroxyacetone. The experiments indicate, on the contrary, that in the presence of an excess of insulin, dihydroxyacetone is not converted into glucose to a very large extent.

EXPERIMENTAL.

The method employed for the determination of dihydroxyacetone is similar to that described in another paper. This method is based upon the property of yeast to adsorb the glucose in the blood or from dilute solutions while dihydroxyacetone remains in solution.

The reduction value was determined in all cases by the Hagedorn-Jensen⁹ method. By this method the monomeric form of dihydroxyacetone had a reducing power of 160 as compared with glucose (100). The reducing power of the solution was determined before and after adsorption with yeast and the glucose was estimated by difference.

All rabbits were of approximately the same weight, about 2 kilos. The animals were allowed to fast for 2 days. On the 3rd day the animal received a subcutaneous injection of iletin (Lilly). The quantity of insulin varied between 15 and 30 units. It was, however, observed that these variations in the quantity of insulin had little influence on the effect of the dihydroxyacetone administered subsequently.

In view of the fact that some animals showed temporary recovery spontaneously, the procedure was adopted of allowing the animals to remain in coma for 20 to 30 minutes before beginning the administration of dihydroxyacetone. The dihydroxyacetone was administered either subcutaneously or intravenously. It was administered either in a single dose or fractionally in small doses. The latter procedure was adopted for the following reason: As is seen from Experiments 8 and 9, after the administration of the large doses the recovery was followed by a relapse into coma with convulsions. It was hoped that by repeated injections of dihydroxyacetone the animal might be maintained without coma and convulsions long enough to permit the natural mechanism of sugar metabolism to be restored to normal.

The blood for analysis was withdrawn directly from the heart in view of the fact that larger quantities were needed for total analysis than could conveniently be obtained from the ear vein.

⁹ Hagedorn, H. C., and Jensen, B. N., *Biochem. Z.*, 1923, cxxxv, 46.

SUGAR METABOLISM. LACTOSE, GALACTOSE, AND XYLOSE.

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(Received for publication, August 2, 1928.)

In the study of the details of the metabolism of individual sugars, it is important to have a method for the estimation of the content, in the blood, of the foreign sugar in the presence of glucose. The usual methods for the separation of individual sugars are at best only first approximations, and in application to such conditions as are met with in blood analysis they are very imperfect indeed. Hence, any improvements in the methods of estimation of the concentration in the blood of foreign sugars in the presence of glucose is of great importance for many physiological and clinical studies. In recent years Corley¹ has made an effort to attain this end by the use of the method of fermentation. He realized, however, the shortcomings of the method. More recently Somogyi² has discovered that a suspension of yeast cells possesses the power of selectively adsorbing glucose and other fermentable sugars. This was confirmed by Raymond and Blanco³ who found that the adsorbing power is selective for glucose, mannose, and fructose while the other monoses are not adsorbed. An attempt was therefore made to apply this method of adsorption to the study of the concentration in the blood of foreign sugars after they had been administered. Three foreign sugars have thus far been studied: lactose (Table I), galactose (Table II), and xylose (Table III). Each sugar was administered in three different ways, namely orally, subcutaneously, and intravenously, and in each case 2.0 gm. were given.

¹ Corley, R. C., *J. Biol. Chem.*, 1926, lxx, 521; 1927, lxxiv, 1; 1928, lxxvi, 31.

² Somogyi, M., *Proc. Soc. Exp. Biol. and Med.*, 1926-27, xxiv, 320; *J. Biol. Chem.*, 1927, lxxv, 33.

³ Raymond, A. L., and Blanco, J. G., *J. Biol. Chem.*, 1928, lxxix, 649.

The results were as follows:

1. *Lactose* (Table I).

A. Oral.—No lactose was found in the blood. The increase of glucose in the blood was considerable.

B. Subcutaneous.—There was present in the blood from 0.082 to 0.180 gm. of lactose per 100 cc. The concentration varied from animal to animal but remained reasonably constant in the same animal. The glucose concentration increased somewhat during the time of the experiment, which lasted 210 minutes.

TABLE I.
Lactose.

In each case 2 gm. of lactose were administered. The results are expressed in mg. of sugar per 100 cc. of blood.

Experiment No.		A. Oral.					B. Subcutaneous.					C. Intravenous.				
		Before.	After:				Before.	After:				Before.	After:			
			30 min.	90 min.	150 min.	210 min.		30 min.	90 min.	150 min.	210 min.		15 min.	30 min.	90 min.	150 min.
I	Glucose.	132	180	218	183	145	128	175	247		376	146	170	147	141	140
	Lactose.		0	0	0	0		166	180	124	136		528	328	242	144
II	Glucose.	144	163	325	314	263	118	141	144	167	205	130	184	595	209	196
	Lactose.		0	0	0	0		106	126	114	116		480	337	122	68
III	Glucose.	146		149	153	188	157	201	167	162	187	138	176	265	190	151
	Lactose.			0	0	0		118	146	154	150		448	302	111	82
IV	Glucose.	110	152	145	165		126	143	203	230	223					
	Lactose.		0	0	0			82	118	120	114					
V	Glucose.	154	205	200	230											
	Lactose.		0	0	0											

C. Intravenous.—The concentration of lactose was greatest after 15 minutes and then gradually dropped. It is noteworthy that in the early phases of the experiment the concentration of glucose also was increased, but after 150 minutes it generally was restored to its normal value. It is also noteworthy that in one experiment after 30 minutes the total sugar concentration was higher than could be accounted for by the injected lactose, and in this case a mobilization of glucose might have been produced by the lactose.

2. Galactose (Table II).

A. Oral.—No galactose was found in the blood and the concentration of glucose varied within normal limits.

B. Subcutaneous.—The concentration of galactose in the blood, with one exception, showed a gradual decline from the initial value. The concentration of glucose showed an increase, though not so high as in the case of lactose, but after 150 minutes in some experiments there was noted a drop to below normal.

TABLE II.

Galactose.

In each case 2 gm. of galactose were administered. The results are expressed in mg. of sugar per 100 cc. of blood.

Experiment No.		A. Oral.					B. Subcutaneous.					C. Intravenous.				
		Before.	After:				Before.	After:				Before.	After:			
			30 min.	90 min.	150 min.	210 min.		30 min.	90 min.	150 min.	210 min.		15 min.	30 min.	60 min.	150 min.
I	Glucose.	143	147	132	175		130	130	151	102	102	157		320	192	205
	Galactose.		0	0	0			140	140	136	100			330	270	120
II	Glucose.	150	230	200	184		95	95	152	180	189	142	270	180	110	120
	Galactose.		0	0	0			120	118	95	60		510	440	333	200
III	Glucose.	123	136	130	125		105	151	151	146	133	139	148	290	201	148
	Galactose.		0	0	0			110	110	93	115		480	390	260	155
IV	Glucose.	130	180	144	132	124	138	146	163	108	107					
	Galactose.		0	0	0	0		129	120	85	64					

C. Intravenous.—The concentration of galactose in the blood reached as high as 0.500 gm. per 100 cc. of blood and dropped continually from the initial value. The concentration of glucose was increased much above the normal and persisted so even after 150 minutes. It is possible that in this case also the injection of the foreign sugar caused a mobilization of glucose.

3. Xylose (Table III).

A. Oral.—Xylose showed a different behavior from that of lactose and of galactose. Whereas the other two sugars did not seem to be adsorbed from the gastrointestinal tract, xylose entered

into the blood even after administration orally. It was also peculiar that the concentration of xylose in the blood seemed to increase with time and often was higher after 210 minutes than after 30 minutes. The concentration of blood glucose showed a slight drop soon after the injection, and the drop generally was followed by a rise which persisted even after 210 minutes.

B. Subcutaneous.—The results of the experiments under these conditions were not uniform. The xylose content in the blood was on the average about 0.150 gm. per 100 cc., and the concentration remained fairly constant during the 210 minutes of the experiment.

TABLE III.

Xylose.

In each case 2 gm. of xylose were administered. The results are expressed in mg. of sugar per 100 cc. of blood.

Experiment No.		A. Oral.					B. Subcutaneous.					C. Intravenous.				
		Before.	After:				Before.	After:				Before.	After:			
			30 min.	90 min.	150 min.	210 min.		30 min.	90 min.	150 min.	210 min.		15 min.	30 min.	45 min.	105 min.
I	Glucose.	160	141	139	200	320	148	125	118	91	121	138	173	263	262	268
	Xylose.		81	102	104	102		121	134	130	81		182	182	195	164
II	Glucose.	165	148	116	250	280	139	97	54	74	92	151	100	230	280	290
	Xylose.		70	109	113	115		111	168	177	168		380	280	250	190
III	Glucose.	159	135	116	130	147	143	175	172	173	190	156	108	290	310	280
	Xylose.		52	80	102	122		113	159	170	141		290	290	265	205
IV	Glucose.						139	148	182	211	298					
	Xylose.							97	141	150	132					

The concentration of glucose varied with the animal. In two out of four experiments, there was noted a drop from the normal concentration which persisted during the time of the experiment, namely, 210 minutes. In two other cases the glucose concentration remained increased above normal.

C. Intravenous.—The highest concentration of xylose in the blood under these conditions of experiment was 0.380 gm. per 100 cc. of blood. The concentration showed a gradual decline. The concentration of blood glucose in two cases out of three showed a drop lasting only a short interval, followed by a persistent rise.

In a third case the rise occurred immediately. Thus it seems as if intravenous administration of each sugar causes a mobilization of glucose.

If these results are compared with those of Corley, there are observed certain disagreements; namely, after oral administration of galactose Corley found it in the blood, whereas in the experiments here reported no galactose was found in the blood. After intravenous injections of the same sugar, Corley, contrary to the observations here reported, found no increase in the concentration of glucose in the blood.

The results of Corley's experiments with xylose harmonized in a general way with ours.

It is realized that the results thus far obtained have only a tentative character and they are reported at this date principally to call attention to the method by which foreign sugars can be conveniently estimated in the blood.

I wish to express my gratitude to Dr. P. A. Levene for his help and encouragement throughout this work.

EXPERIMENTAL.

All experiments were performed on normal rabbits of about 2000 gm. weight. They were not allowed to fast. 1 hour before the beginning of the experiment the animal received an injection of 0.05 gm. of amytal per kilo of body weight. This dose has been known not to produce hyperglycemia and this generally accepted view was confirmed experimentally by us.

Three series of experiments were performed with each sugar. In one series the sugar was administered orally, in the second subcutaneously, and in the third intravenously. 2.0 gm. were administered in each experiment.

The blood sugar estimation was made in the following way. 1.2 cc. of blood were withdrawn from the heart of the animal prior to the injection and ingestion of the foreign sugar and other portions of the same size at intervals after administration. Of these samples 0.1 cc. was employed for the estimation of the total reducing power. 1.0 cc. was employed for the differential estimation of glucose and the other reducing substances. From this quantity of blood the glucose was removed by adsorption by a yeast sus-

pension. The power of yeast to adsorb or to absorb glucose was discovered by Somogyi.² The details of the procedure were the following. 1.0 cc. of blood was mixed with 7 cc. of a 10 per cent suspension of bakers' yeast (Fleischmann). 1 cc. of a 10 per cent solution of tungstate and 1 cc. of 0.66 N sulfuric acid were added, and the suspension was well shaken and allowed to stand for 5 minutes. The mixture was then centrifuged, the supernatant liquid filtered, and 1 cc. of it was taken for a sugar estimation according to Hagedorn and Jensen.⁴ This value represents the sum of the values of the non-glucose reducing power and of the foreign sugar. Thus the values of glucose and of foreign sugar can be calculated by difference. The non-sugar reducing power of normal blood is equivalent to 0.019 to 0.022 gm. of glucose per 100 cc. of blood. The control samples of the various sugars were added to normal blood and the adsorbing power of yeast suspension for these sugars under these conditions was tested. The results were always negative.

The results are summarized in Tables I to III, the unit being mg. of the sugar per 100 cc. of blood.

⁴ Hagedorn, H. C., and Jensen, B. N., *Biochem. Z.*, 1923, cxxxv, 46.

EFFECT OF ROENTGEN RADIATION ON SOLUTIONS OF TYROSINE AND CYSTINE.

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One of the outstanding problems, in any attempt to analyze the ultimate nature of the effect upon living tissues which is being produced by x-rays, is the elucidation of the effect upon the body proteins.

Two main lines of attack of this problem present themselves; first the denaturing effect upon the different pure proteins and second the possible effects upon the structure of the different amino acids. In this paper we present the first results of some experiments which we are conducting along the second of these two lines. As far as we know the effect on amino acids has not been studied before.

The radiation for these experiments was obtained from a water-cooled deep therapy Coolidge tube while patients were being treated; 200 kilovolts and 30 milliamperes were used. The solutions were sealed in glass tubes 15 cm. long, $1\frac{1}{2}$ cm. in diameter, and $1\frac{1}{2}$ mm. in thickness (about 15 cc. in each tube). Two of these tubes were prepared at the same time, one being used as a control by the following arrangement. One was placed inside a lead cylinder with walls, cap, and bottom 3 mm. in thickness. This lead protection was sufficient to prevent any appreciable amount of radiation from reaching the solution. The other tube was placed inside an aluminum container with walls, 0.3 mm. in thickness. Thus only a small amount of the Roentgen radiation was stopped by the aluminum while visible radiation was excluded and the temperature of the two tubes was equalized. The lead and aluminum container were placed inside a large metal cylinder in which the Roentgen tube was installed. The distance from the target to the center of the tubes was 57 cm.

The tyrosine was recrystallized by the method of Folin. Folin's method for the determination of tyrosine by means of the phenol reagent was used. Both the recrystallization and the determination were used in the form recently described by Folin and Ciocalteu (1).

The first solution of tyrosine (Solution A) used contained 1 mg. of tyrosine in 1 cc. of a 2 N sulfuric acid solution. The sulfuric acid was present to increase the solubility of the tyrosine and the keeping qualities of the solution. The first pair of tubes was radiated for 27 hours. Only about 2 per cent of the tyrosine was changed. The time was then increased to 156 hours for the next pair of tubes and 14 per cent of the tyrosine was changed. The change thus seemed to be about proportional to the time of radiation.

A 10 times weaker solution of tyrosine (Solution B) was then made up, containing 0.1 mg. of tyrosine per cc. in a 2 N sulfuric acid solution. An exposure of 26 hours and 50 minutes resulted in a change of 17 per cent of tyrosine, which corresponds to almost the same absolute amount as was changed in the stronger solution exposed for about the same time. This indicates that a certain amount of radiation would change a certain number of tyrosine molecules almost independently of the concentration.

A third solution of tyrosine (Solution E) was also made up by diluting a part of Solution A 50 times with distilled water, giving a concentration of 0.02 mg. of tyrosine in 1 cc. of 0.04 N sulfuric acid solution. This solution was radiated without aluminum protection in glass-stoppered tubes, 2.5 cm. long, 20 cm. in diameter, and 1.3 mm. in thickness (about 50 cc. in each tube). The results of the radiation of this solution, as well as of Solutions A and B just referred to, are given in Chart 1.

In Solution A, 1 cc. was used for the determination, in Solution B, 10 cc. were used, and in Solution E, 50 cc. In each case the unirradiated sample was used for the standard.

The sum of the experimental errors in the exposures amounts to about 5 per cent, while those of the tyrosine determination are approximately 2 per cent. This error is increased by the development of a brown color caused by the decomposition of the tyrosine, the more decomposition the greater the error introduced in the determination.

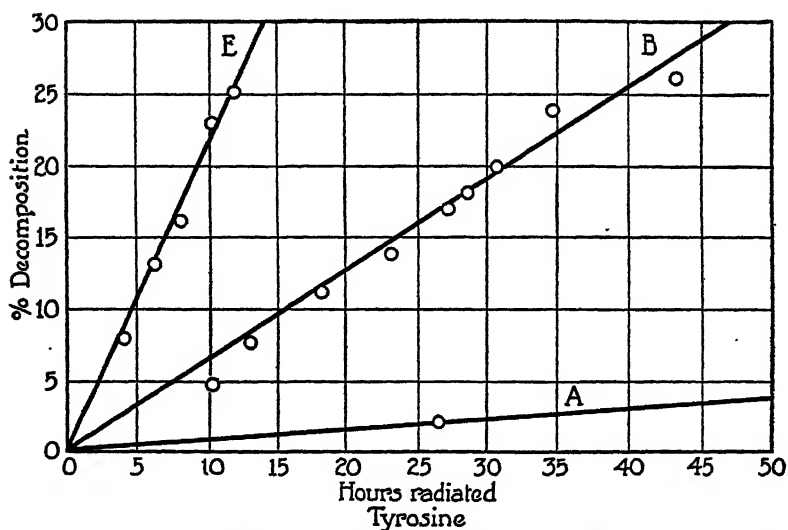


CHART 1. Showing that the amount of tyrosine changed is proportional to the amount of radiation.

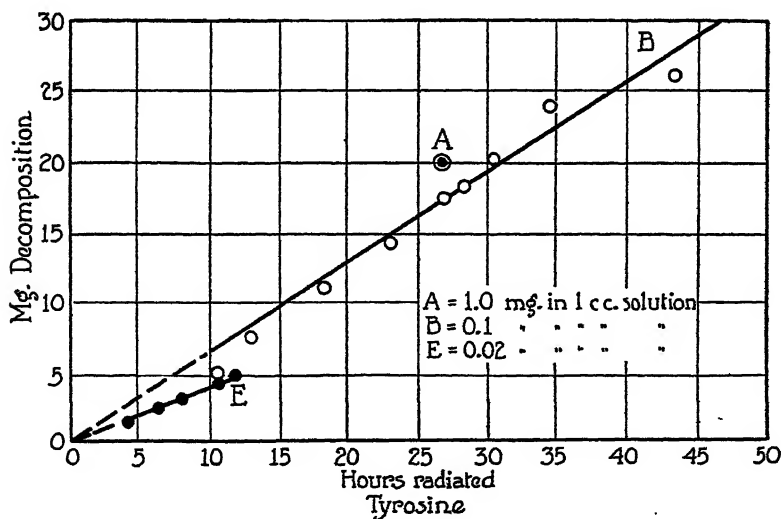


CHART 2. Showing the influence of the concentration of the tyrosine solution upon the amount of tyrosine changed. Lines drawn by inspection.

An analysis of Chart 1 shows that the change is directly proportional to the dosage. The influence of the concentration upon the amount of tyrosine that is being altered is slight and is graphically presented in Chart 2. When the concentration is reduced 50 times the efficiency of the x-rays to decompose the tyrosine is reduced only about to one-half.

We have also determined the influence of radiation on a pure tyrosine solution without any sulfuric acid. The same concen-

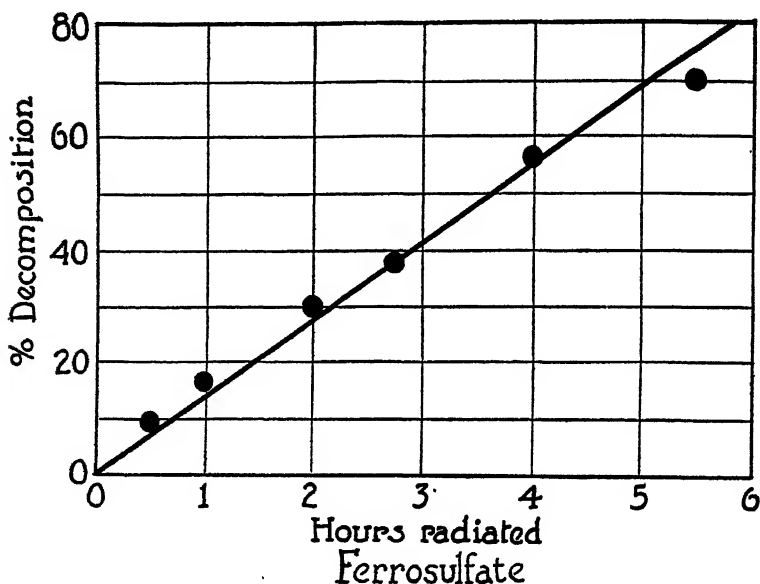


CHART 3. Showing the oxidation of ferrosulfate proportional to the amount of radiation.

tration was used as in the weakest tyrosine solution (Solution E). When this solution was radiated 4.5 and 7.5 hours, 9 per cent and 15 per cent respectively were changed. This is in practically quantitative agreement with the change obtained in the acid solution. The sulfuric acid evidently does not appreciably influence the rate of the change.

The sequence of the events, therefore, seems to be a primary ionization of water molecules and a following transference of energy

from the ions to the tyrosine molecules. The phenol structure is evidently changed.

In order to find out how much radiation had been absorbed by the solution, we used a method for measurement of dosage that recently has been proposed by Fricke and Morse (2). They found that FeSO_4 in 0.8 N H_2SO_4 solution is oxidized to $\text{Fe}_2(\text{SO}_4)_3$ by Roentgen radiation. They also found that the amount oxidized was directly proportional to the Roentgen dose and that 56.7 kilo Roentgens (K.R.) are required for the oxidation of all the FeSO_4 in 0.001 N solution. We radiated such solutions in the same way as the tyrosine solutions had been radiated, and measured the amount of FeSO_4 oxidized with an electrometric titration method similar in principle to the one used by Fricke and Morse with the exception that we took the end-point as the place where there was a large change in E.M.F. according to the method suggested by Kolthoff and Furman (3). The results of these experiments are shown in Chart 3. From these measurements we find that the solution on an average absorbs 7.86 K.R. per cc. per hour.

According to these findings all the tyrosine in Solution E should be changed if irradiated for 47 hours and 16 minutes or by 372 K.R.; thus, 0.02 mg. of tyrosine will be changed by 372 K.R. or 0.0000538 mg. of tyrosine by 1 K.R. This gives 6.06×10^{20}

$\times \frac{538}{181} \times 10^{-7}$ or 1.80×10^{14} molecules for 1 K.R. As 1 R. produces $\frac{10^{10}}{4.77}$ pair of ions per cc. of air, it ought to produce about 1000

times as many in the solution which has an effective atomic number about equal to that of air. 1 K.R. should therefore produce about $2.1 \times 10^9 \times 10^6$ pair of ions. The number of molecules (M) changed per pair of ions (N) should be, $\frac{1.80 \times 10^{14}}{2.1 \times 10^{15}}$ or about

$\frac{1}{12} \left(= \frac{M}{N} \right)$. When a 50 times as concentrated solution is irradiated, $\frac{M}{N}$ would become about $\frac{1}{6}$, for FeSO_4 $\frac{M}{N}$ is about 5.

According to these findings it is possible that an appreciable amount of tyrosine may be changed in the body when a patient is exposed to an erythema dose which amounts to more than 1 K.R. per cc. in a considerable volume. However, other body

substances may be more reactive than tyrosine and may utilize the available ionization energy involved.

A solution of 0.1 mg. of cystine in 1 cc. of 1 N H_2SO_4 was exposed for as much as 95 hours (770 K.R.). No measurable change was, however, noticed when the colorimetric method (4) was used to determine the cystine present.

SUMMARY.

When tyrosine in a weak aqueous solution is exposed to Roentgen radiation it is changed in regard to the phenol group. The amount changed is proportional to the dose of radiation absorbed. It varies only slightly with the concentration, the result of high dilution being a small decrease in the efficiency of the radiation. About 0.01 mg. of tyrosine in a 0.002 per cent solution is changed by 186 K.R. $\frac{M}{N}$ is approximately $\frac{1}{12}$.

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THE IRON AND MANGANESE CONTENT OF FEEDING STUFFS.*

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That the inorganic elements play an important rôle in plant and animal metabolism has long been recognized. However, it is only in comparatively recent years that the importance of some of the less obvious of these elements has been realized and attempts made to determine their function in the animal organism (1-5). For example, the wide-spread occurrence of manganese in the tissues of animals (6-9) is an indication that it probably serves a useful purpose there. Direct evidence of the nutritional value of manganese has been furnished through various feeding experiments. Richet, Gardner, and Goodbody (10) have demonstrated that manganese, if administered in small amounts, produces a favorable effect upon the growth of dogs. More recently McCarrison (11) reported that manganese, when ingested by young rats to the extent of 1 part in 617,700 parts of ration, was beneficial in promoting growth. However, when the manganese was increased to 1 part in 12,000, a retarding influence upon the rate of growth became evident after 32 days.

In view of the demonstrated function of manganese in animal metabolism, a knowledge of the content of this element in the various feeding stuffs is highly desirable. A few papers (12-15), have already been published on this subject, but many of the materials commonly consumed by farm animals are not included in these papers.

Unlike manganese, iron has been recognized for many years as important in nutrition. Consequently, the lack of analytical

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data with regard to the iron content of various feeding stuffs is rather surprising. Henry and Morrison (16) in their tables of the mineral matter in representative feeding stuffs give the iron content for only one-half of the materials listed. Additional analyses have been reported (12-14, 17) but the number of samples was rather limited.

In this paper will be presented the manganese content of 54 feeding stuffs and the iron content of 51 of the same samples.

EXPERIMENTAL.

Preparation and Ashing of Samples.—The feeding stuffs were either obtained from the local market or were taken directly from the field. After being dried, all coarse materials were reduced to a finely divided state, care being taken to avoid the introduction of iron or manganese. The samples were ashed in a muffle furnace at a cherry-red heat until all carbon had been oxidized. Samples which were extremely hard to ash were first burned at a somewhat lower temperature and the partially oxidized mass was then extracted with water and filtered. After the residue was dried it was ignited to a carbon-free ash.

Methods of Analysis.

Iron.—The iron analyses were made according to the ferric thiocyanate method given in Standard Methods for the Examination of Water and Sewage by the American Public Health Association (18). Precautions with respect to dishes and reagents described in a previous publication (19) were observed.

In order to secure representative samples from the various feeds 2 to 4 gm. portions were usually ashed. The ash was dissolved in HCl (1:4) made to a definite volume and aliquots assumed to contain from 0.1 to 0.3 mg. of iron were taken for analysis. In the analysis of some of the samples it was thought that it might be desirable to remove the silica by evaporating the solution to dryness, but no color could be obtained with a sample which had received such treatment. No attempt was therefore made to remove the silica.

The high concentration of phosphates in the cereals frequently prevented the full development of the thiocyanate color. This

difficulty was overcome by oxidizing the HCl solution of the ash with 30 per cent H_2O_2 , a reagent which had been found successful in such cases by Dr. C. A. Elvehjem of this laboratory.

All analyses were run in triplicate and to one of them was added 0.1 mg. of iron for recovery. If a satisfactory recovery of the added iron was obtained it was felt that the iron content

TABLE I.

Detailed Data Illustrating Procedure in Determination of Iron in Representative Samples of Feeding Stuffs.

Material.	Weight of sample.	Weight of sample in aliquot.	Fe in stand-ard.	Readings.		Fe in sample.	Recov-ery of added Fe.	Fe
				Stand-ard.	Ali-quot.			
	gm.	gm.	mg.	mm.	mm.	mg.	per cent	per cent
Alfalfa hay.	2	0.8	0.1	25	17.8	0.351		0.0176
	2	0.8	0.1	25	18.0	0.348		0.0174
	2*	0.8	0.2	20	22.3	0.449	99	
Corn, yellow.	2	2.0	0.1	25	27.7	0.090		0.0045
	2	2.0	0.1	25	28.1	0.089		0.0045
	2*	2.0	0.2	20	20.5	0.195	106	
Corn stover.	4	1.6	0.2	20	26.8	0.373		0.0093
	4	1.6	0.2	20	26.6	0.376		0.0094
	4*	1.6	0.2	20	21.0	0.476	102	
Rape.	2	0.8	0.2	20	20.4	0.490		0.0245
	2	0.8	0.2	20	20.6	0.485		0.0243
	2*	0.8	0.2	20	17.0	0.588	101	
Tankage.	1	0.2	0.1	25	26.1	0.483		0.0483
	1	0.2	0.1	25	25.9	0.479		0.0479
	1*	0.2	0.1	25	19.2	0.571	90	

* A quantity of the standard iron solution containing 0.1 mg. of Fe was added to this sample before ashing.

of the sample was being accurately determined. There was no appreciable difference whether the iron was added prior to ashing or just before oxidizing the solution with permanganate.

The detailed data for five of the 51 samples are given in Table I. These five samples are representative of the different classes of feeding stuffs and include materials which range from low to high

in iron content. The recovery of added iron is from 90 to 106 per cent and shows the reliability of the method. The same procedure was followed in the analysis of all the other samples. If a satisfactory recovery was not obtained the first time, the analysis was repeated with particular attention to the ashing and to the oxidation of the hydrochloric acid extract.

Manganese.—This element was determined by the procedure given in Methods of Analysis of the Association of Official Agricultural Chemists (20). Certain modifications in procedure and precautions with respect to reagents and dishes have been described in a previous publication (21).

Samples high in calcium frequently showed a separation of CaSO_4 while the solution was being evaporated for the removal of chlorine. In such cases it was necessary to shake the beaker continuously to prevent spattering and loss of manganese. A satisfactory reading of the colorimeter in the presence of this precipitate was impossible and therefore it was removed by filtering through a layer of manganese-free asbestos in a Gooch crucible. The residue was washed with small portions of water until the volume of the filtrate was approximately 30 cc.

In a few instances during oxidation with KIO_4 the permanganate color faded as the volume was reduced. This difficulty was overcome by the addition of a few drops of sirupy phosphoric acid to the boiling solution.

As in the analysis of iron, triplicate determinations, one of which was a recovery, were made upon each feed. The percentage recovery was slightly lower when the manganese was added before the sample was ashed. The lowest recoveries were obtained with those samples which yielded large amounts of ash. It is probable that the loss was due to occlusion of the added manganese in the silica and other insoluble compounds. Samples of such size were taken that the color could be compared with that of a standard containing from 0.1 to 0.25 mg. of manganese. When concentrations greater than these are present, the KIO_4 may fail to develop completely the permanganate color.

Table II illustrates the procedure in detail for the determination of manganese in five feeding stuffs. The difficulties encountered in the analyses of these feeds are representative of those met with in the analyses of all the materials. The recovery was better than

90 per cent for each of these samples. In only eight analyses was the recovery less than 90 per cent.

DISCUSSION.

The amounts of iron and manganese per kilo of dry matter for all the samples are given in Table III.

TABLE II.

Detailed Data Illustrating Procedure in Determination of Manganese in Representative Samples of Feeding Stuffs.

Material.	Weight of sample.	Mn in stand-ard.	Readings.		Mn in sample.	Recov-ery of added Mn.	Mn
			Stand-ard.	Sample.			
	gm.	mg.	mm.	mm.	mg.	per cent	per cent
Alfalfa hay.	2.5	0.148	20	16.5	0.179		0.0072
	2.5	0.148	20	16.8	0.176		0.0071
	2.5*	0.247	15	13.7	0.271	94	
Corn, yellow.	15	0.148	20	37.0	0.080		0.00053
	15	0.148	20	36.0	0.082		0.00054
	15*	0.148	20	16.4	0.181	100	
Cottonseed meal.	15	0.247	15	12.2	0.304		0.0020
	15	0.247	15	12.6	0.294		0.0020
	15*	0.247	15	9.4	0.394	96	
Oat straw.	2	0.148	20	17.0	0.175		0.0087
	2	0.148	20	17.0	0.175		0.0087
	2*	0.247	15	13.7	0.271	96	
Sugar beet roots.	1	0.148	20	14.8	0.200		0.0200
	1	0.148	20	14.0	0.211		0.0211
	1*	0.247	15	12.5	0.296	92	

* 0.099 mg. of Mn was added to this sample.

Iron.—The iron content in the 51 feeds ranged from 8.9 mg. per kilo for polished rice to 750 mg. for coconut oil meal. 80 per cent of the samples contained between 40 and 500 mg. per kilo. The average for all the samples was 203 mg. per kilo.

The variations observed in a single feed were enormous. For example, five samples of alfalfa varied from 126.5 to 681.8 mg. per

684 Fe and Mn Content of Feeding Stuffs

TABLE III.
Iron and Manganese Content of Feeding Stuffs.

Material.	Fe in dry matter (100%).	Mn in dry matter (100%).
	<i>mg. per kg.</i>	<i>mg. per kg.</i>
Alfalfa hay.....	174.7	71.2
“ “ first cutting (Wisconsin).....	242.8	46.0
“ “ second “ “.....	126.5	47.9
“ “ first “ (Colorado), Sample 1.....	681.8	27.0
“ “ “ “ “ “ 2.....	497.5	44.0
Barley.....	65.1	19.0
Blood meal.....	512.5	8.9
Bluegrass.....	425.5	59.0
Brewers' dried grain.....	170.9	38.3
Buckwheat feed.....	69.8	89.3
“ hulls.....	99.3	97.2
Coconut oil meal.....	750.0	87.0
Corn, yellow.....	44.8	5.4
“ gluten meal.....	183.9	5.9
“ oil cake meal.....	182.9	15.0
“ silage.....	362.9	34.8
“ stover.....	93.7	53.7
Cottonseed meal.....	53.9	19.9
Distillers' dried grain.....	360.3	24.7
Emmer.....	51.7	35.2
Gluten feed.....	463.0	26.9
Groats.....	36.8	37.4
Hominy feed.....	94.4	16.4
Ivory nut meal.....	465.0	14.7
Kaffir grain, white.....	39.1	15.9
Linseed meal.....	105.8	49.4
Meat meal.....	241.5	18.7
“ and bone meal.....	438.5	10.0
Millet, Japanese.....	58.7	35.6
Oats.....	42.3	31.8
Oat straw.....	61.3	87.1
Rape.....	243.9	51.1
Rice, brewers'.....	11.0	16.8
“ polished.....	8.9	12.0
Rye, Sample 1.....	57.4	50.1
“ “ 2.....		32.3
Sorghum fodder.....	54.3	41.1
Soy bean seed.....	59.5	29.5
“ “ hay.....	304.0	84.1

TABLE III—*Concluded.*

Material.	Fe in dry matter (100%).	Mn in dry matter (100%).
	mg. per kg.	mg. per kg.
Sugar beet roots (field).....	53.5	205.7
“ “ tops (field).....	178.7	712.4
“ “ “ (greenhouse).....	478.5	102.9
Sweet clover hay.....	212.8	46.1
Tankage.....	480.8	14.3
Timothy hay.....	30.2	32.0
Vetch hay.....	354.5	106.5
Wheat.....	46.3	54.5
“ bran, Sample 1.....	162.8	140.4
“ “ “ 2.....		140.4
“ gluten.....	71.0	27.4
“ middlings, flour.....	120.3	129.6
“ “ standard.....	192.4	155.8
“ straw, Sample 1.....	71.0	24.1
“ “ “ 2.....		30.8

kilo. The first cutting of alfalfa contained 242.8 mg. per kilo whereas the second cutting from the same plot of land contained only 126.5 mg. Apparently the available iron supply was being rapidly diminished by the growing plant.

The average iron content arranged in descending order and expressed as mg. per kilo of dry matter for some of the principal classes of feeding stuffs are: 4 animal products, 418.4; 4 green forage crops, 331.7; 8 legume hays, 324.3; 19 by-product concentrates, 189.6; 3 cured forage feeds from grasses and cereals, 59.4; 9 grains and seeds, 51.6; 2 straws, 43.7. The group containing the grains and seeds showed the least variation, the limits for this group being 39.1 and 65.1 mg. per kilo. The percentage of iron was greater in the by-product concentrates than in the materials from which they are manufactured.

Manganese.—The amount of manganese as given in Table III varied from 5.4 mg. per kilo for yellow corn to 712.4 mg. for sugar beet tops. Of the entire number of samples 90 per cent contained from 10 to 150 mg. per kilo. The average for all the samples was 63 mg. per kilo.

The manganese in different samples of the same feed varied widely. Sugar beet tops grown in the field contained 712.4 mg. per

kilo as compared to 102.9 mg. per kilo in a sample grown in a greenhouse. The manganese in five samples of alfalfa hay grown on different soils varied from 27 to 71.2 mg. per kilo. In contrast to the variations in iron, the manganese figures for the first and second cuttings were much alike, 46 and 47.9 mg. per kilo respectively. One sample of rye contained 32.3 mg. per kilo, another contained 50.1 mg. but two samples of wheat bran were found to contain the same amount of manganese.

From the standpoint of manganese, the group containing green forage crops far surpasses any of the other groups of feeding materials. The average for this group was 231.4 mg. per kilo, whereas the next highest group showed an average manganese content of only 59.1 mg. The grains and seeds were not found to be as rich in manganese as the by-products obtained from them. These facts indicate a concentration of the element in the seed coat.

Comparison of Distribution of Iron and Manganese.

In previous publications (21, 22) it was reported that the iron content of human foods was from 2 to 25 times as great as the manganese. In the animal feeding stuffs listed in Table III, the average iron content is about 3 times that of the manganese. In the animal products, such as tankage, the iron is more than 30 times as abundant as the manganese. In the grains and seeds nine of the 51 samples contained more manganese than iron. A change in iron content is not related to a change in manganese. Of the five samples of alfalfa the one containing the greatest amount of iron contained the least amount of manganese.

SUMMARY.

The manganese content of 54 samples of a wide variety of feeding stuffs and the iron content of 51 of the same samples are given.

The figures (dry basis) range from 5.4 to 712.4 mg. per kilo for manganese and from 8.9 to 750 mg. for iron. Great variations for both elements were observed in different samples of the same feeding stuff.

The average iron content was 3 times the average manganese content. In only nine samples did the manganese exceed the iron.

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OXIDATION-REDUCTION SYSTEMS OF BIOLOGICAL SIGNIFICANCE.

I. THE REDUCTION POTENTIAL OF CYSTEINE: ITS MEASUREMENT AND SIGNIFICANCE.

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It is well known that tissue extracts or suspensions of cells establish a rather strong negative electric potential under anaerobic conditions at indifferent electrodes such as platinum, gold, or mercury. An observation of this sort was first made by Gillespie on bacteria; he inaugurated an interpretation based on the concepts of reduction potentials. The success met on application of the theoretical fundamentals of oxidation-reduction potentials (hereinafter to be referred to by us as "redox" potentials) as developed by Ostwald, Nernst, Haber, Luther, Fredenhagen, Leblanc, Peters, and others, to organic redox systems in the hands of Büllmann and more particularly W. M. Clark has given great impetus to more elaborate study of physiological materials. Among those investigators attempting to relate their studies of biological material to those of the reversible systems it may be sufficient to mention W. M. Clark, Conant, Dixon, Quastel, Tunnicliffe, Drew, Fleisch, E. N. Harvey, Kendall and Nord, Needham and Needham, Rapkine and Wurmser, Thunberg, Voegtlin, Johnson and Dyer.

The concept of a definite, finite redox potential and of a definite, finite redox system is limited to reversible systems and moreover to reversible systems at equilibrium. An attempt to apply the theory of redox potentials to physiological materials is met with the difficulty that oxidation in tissues is, on the whole, an irreversible process impossible of ending in a state of equilibrium, such equilibrium being contradictory to life. Is there then sufficient

reason to attempt to correlate biological reduction potentials with those inanimate redox systems capable of exact thermodynamic treatment?

There is one condition which permits this correlation. When a tissue, naturally aerobic, is deprived of its oxygen supply and when it has reduced the last small quantities of available oxygen as far as it can, then the reducing substance of the tissue acts on other of the tissue compounds and reduction goes on until a definite equilibrium is established. This equilibrium to be sure means death, but the final potential reached after its establishment must in some way be related to the chemical composition of the tissue in its living state. The potential established after withdrawal of oxygen supply may, therefore, be different for different kinds of cells and in addition be related to the individual properties of these cells when living.

Now let the problem be complicated by the presence of a normal oxygen environment and by the continuous, irreversible flow of oxidation processes consequent upon it. If there be some reversible redox system in the tissues, what conditions are essential to constancy of ratio of oxidized to reduced component of that particular redox system describable in terms of potential? A dynamic process at a constant level will be existent only when there is a constant ratio of reducible substances (say activated oxygen) furnished from latent sources (say air) to oxidizable substances (such as active glucose) from latent sources (such as glycogen). In so far as reaction velocities are involved in establishment of the constant level of which we speak, it is not necessarily true that any reversible redox system—natural or artificially added—would be kept at the same level of potential. There is, then, in the presence of oxygen, an important divergence from a tissue or a system at equilibrium.

For sake of greater clarity let us make a system of the kind we consider and let us examine its behavior. We add a definite amount of methylene blue to a measured quantity of tissue extract and maintain a constant oxygen pressure. We find in time that the dyestuff is partially reduced; a methylene blue-methylene white system has been established with its components in definite ratio. If we vary any of our quantities—dye, tissue extract, or oxygen—we can create a new methylene blue-methylene white

ratio. When the oxygen pressure is that of air, for instance, the methylene blue is present in a practically completely oxidized state; when the oxygen pressure is very low (as in an anaerobic Thunberg experiment) the methylene blue system is completely reduced; and there must be a range of intermediary oxygen pressures at which a definite ratio of methylene blue and methylene white will be established characteristic for the particular pressure. This is equivalent to the statement that by changing the oxygen pressure we can create a new potential of the methylene blue system.

In complete absence of oxygen the final condition may be considered a true equilibrium. In this case we may expect the potential as indicated by the added reversible dyestuff system to be independent of the particular chemical nature of the dye, provided the concentration of the dye is low enough to work only as an indicator and not to poise the system. In such a case, therefore, it is legitimate to speak of a definite reduction potential of the system as a whole.

When a definite oxygen pressure is maintained, however, no true equilibrium of the whole system will be reached but at best the methylene blue system will be kept in a constant condition. Reduction velocities are involved in establishment of the level of this condition, *e.g.* the velocity with which reduced dye is oxidized by molecular oxygen, and it is not necessarily true, as has been said, that different indicators be at the same potential. Nor is it necessarily true that the system as a whole have a definite potential; if there be more than one redox system present in tissue, each may have its individual potential.

Our problem of the moment, then, is to discover under natural conditions a substance or substances among the constituents of the tissue liquids which behave as the artificially added methylene blue, and which account for those biological reduction potentials, measurable at indifferent electrodes, of which we have spoken. A few systems corresponding to methylene blue have been described in tissues. Such, according to Cannan (1926, 1927), is the echinochrome of *Arbacia* eggs and the hermidin of *Mercurialis perennis*. The cytochrome described by Keilin (1925) and found widely distributed in mammalian tissues and in yeast, is as yet too little understood for certainty of its relationship to

tissue potentials. Biological systems demonstrated to behave like methylene blue are thus seen to be much restricted in their distribution and so of limited moment in the problem we have set ourselves. There is, however, a certain group of substances found ubiquitously in animals which are related to the methylene blue system in a peculiar way not yet fully understood and appreciated and which are moreover highly responsible for the potentials of noble metal electrodes dipping into tissue liquid. These substances are the sulfhydryl bodies. The most accessible representative of them is cysteine, and the one probably most responsible for the behavior of tissue is the glutathione of Hopkins. This substance was previously considered by the discoverer himself as reversibly oxidizable and reducible, and in this respect equivalent to the methylene blue system. This idea, however, was modified by further investigations from Hopkins' laboratory as is told below.

The fact that cysteine (and reduced glutathione and sulfhydryl substances in general) can exhibit a strong reducing power led to an investigation of its potential at a noble electrode. Dixon and Quastel (1923) first attacked this problem; they observed a strong negative potential at an inert electrode dipping into a solution of cysteine or glutathione. Quantitative measurements led to the following formula in which RSH stands for cysteine (or reduced glutathione):

$$E = E_0 - \frac{RT}{F} \ln [\text{RSH}] + \frac{RT}{F} \ln [\text{H}^+]$$

The potential E was measured at electrodes of solid gold; only such electrodes gave well defined values according to these authors. Platinum and gold-plated platinum gave inconsistent, drifting potentials. The massive gold electrode, too, failed of complete satisfaction. The constant E_0 , and this should be emphasized even more than was done by Dixon and Quastel, was a constant only during a single titration experiment in the course of which either pH or cysteine concentration was varied. E_0 varied for different gold electrodes and moreover was variable without recognizable cause for the same electrode in different experiments to an extent of often as much as 60 millivolts.

Beyond the rather unusual difficulties encountered in measuring

the potential, it is both remarkable and interesting to note that the formula is without reference to cysteine's oxidation product, cystine, which may be symbolized by RSSR. The potential, accordingly, of cysteine (and of reduced glutathione as well) is independent of the presence or absence of its oxidation product. Cysteine can be prepared readily from cystine by reduction and cystine from cysteine by a simple oxidation; still they do not constitute a simple reversible redox system. Dixon and Quastel, therefore, justly considered the behavior of these substances as very unusual and in consequence assigned them to a new type of reduction-oxidation system.

The attempts of these authors to explain this behavior do not appear to be wholly successful. They first resort to the plausible hypothesis that there exists a primary oxidation product of cysteine (from which cystine arises only through a secondary irreversible process); they then proceed to the unlikely supposition that this primary oxidation product is always present in *invariable* concentration. This view-point, equivalent to considering the solution at all times saturated with the primary oxidation product, appears to have been abandoned by the authors themselves in a later communication.

This second paper by Dixon (1927) attempts to interpret the potentials from the standpoint of Wieland's theory of hydrogen acceptors. Wieland has shown that palladium black, placed into solutions of many different reductants, becomes charged with hydrogen; the claim is made that cysteine is able to charge the metal electrode with hydrogen but that cystine is not able to withdraw hydrogen from the hydrogen-charged metal. The process of charging the metal with hydrogen, in consequence, must be considered irreversible; the counterbalancing effect of withdrawal, evident in all reversible redox systems, is missing here. The hydrogen charge of the metal, however, is limited by diffusion of the gas out of the metal into the surrounding solution. Equilibrium of metal and hydrogen is reached when the rate of diffusion of hydrogen into the solution from the metal exactly balances the rate of supply of hydrogen for the metal by cysteine. The potential obtained is a hydrogen potential; the electrode works as a hydrogen electrode of a definite gas pressure for each particular environment.

. This is the essence of Dixon's second attempt to explain the unique behavior of the cysteine potential. He went a step further and put his explanation to further test. The rate at which metals can be charged with hydrogen and the rate at which they will release the gas into the surrounding medium may well vary with the nature of the metal; the final potential, therefore, may be made to change by changing the metal electrode. Dixon found, indeed, that mercury gives a potential some 200 millivolts more negative than gold in the same cysteine solution; his ideas received still more support on consideration of the fact that mercury is capable of a much higher hydrogen overvoltage on cathodic polarization than gold. We shall show that these latter arguments of Dixon are faced with serious objection.

Another investigation of the cysteine potential has been made by Kendall and Nord (1926). We have not concerned ourselves with detailed study of the potential established in the presence of indigo carmine after treatment with oxygen or hydrogen peroxide; we shall not present, therefore, a critique of their theoretical treatment of this part of the subject. Suffice it to mention that Kendall and Nord confirmed, in an entirely qualitative way, the observations of Dixon and Quastel that cysteine does give a reduction potential when present alone in pure buffer solutions and that this potential is independent of the concentration of cystine. Kendall and Nord add no explanation of these observations to those already given by Dixon and Quastel.

It is quite unnecessary to emphasize the importance of complete understanding of the cysteine potential, to point out that it may well be correlated with the fundamental problems of tissue respiration. We could not be entirely satisfied with the results of Dixon and Quastel for their measurements suffered from failure of good reproducibility and their considerations, in consequence, were focused upon the findings of single titration experiments. Comparison and analysis of experiments independently performed were rendered difficult and uncertain. We sought, therefore, that method of procedure which brought with it exact reproduction of results, which gave us means of measurement capable of complete control. Such a method must be considered of first importance for an exact understanding of the problem at hand; it may be of service, we hope, in investigation of many closely

allied biochemical questions. We present in the following pages the development of such a method and the observations and considerations growing out of the experiments necessary to its establishment.

Technique.

It ensues from the considerations above that control of oxygen in systems to be investigated is of greatest importance. With many reversible systems a slight amount of this gas is often without effect on the electrode potential; this is particularly true of potentials in positive ranges such as those of quinone-hydroquinone, or ferric-ferrous ions, or ferrocyanide-ferricyanide ions. In other reversible systems, such as dyestuffs, lying in the negative range of potentials, oxygen is of much greater significance. Here, however, the reductant is usually enormously avid for molecular oxygen; the potential measured in presence of a trace of the gas suffers only from change of ratio of reduced to oxidized component consequent upon oxygen combination. This change may be insignificant when a fairly well purified nitrogen atmosphere is supplied, and the redox system is sufficiently poised to undergo no appreciable change of ratio of oxidant to reductant by consumption of the oxygen impurity. In solutions of substances like cysteine, however, even the slightest traces of oxygen have a great effect upon the potential. The reduction potentials of such solutions are always diminished, sometimes enormously, by presence of extremely small quantities of the gas. Spontaneous consumption of traces of oxygen cannot be relied upon in these cases. Cysteine even in presence of iron consumes oxygen with reasonable speed only over a limited range of pH; moreover, even at the optimum pH it appears to fail to remove the last traces of the gas.

The most important goal of the technique was, therefore, to remove oxygen as completely as possible from the cysteine system. This involved simply a supply of carefully purified nitrogen to a carefully sealed apparatus. Nitrogen, usually containing about 0.08 per cent oxygen,¹ from a commercial tank was slowly led through NaOH and then through a Pyrex glass tube

¹ Repeated analyses gave this value. It is lower for this nitrogen tank than that reported by other investigators.

60 cc. long tightly packed with short pieces of copper oxide wire. The copper oxide was previously reduced by hydrogen; this procedure was frequently repeated during the course of the experiments. The tube was maintained in an electric furnace at a temperature of 550–600°. Leads from the combustion tube to the electrode vessel were of copper tubing; connections between metal and metal were soldered; connections between metal and

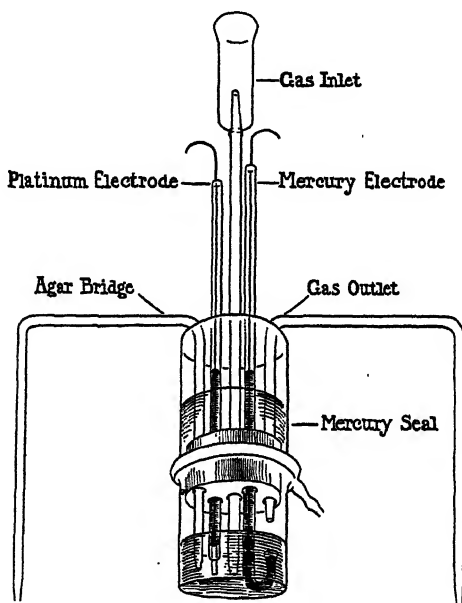


FIG. 1. One of the types of electrode vessels used. Copper tubing attached to gas inlet as in Fig. 2. The upper glass cylinder filled with mercury is held in place by means of solid paraffin.

glass were sealed with De Khotinsky cement. The end of the copper tube was connected to the gas inlet of the electrode vessel by a mercury-sealed arrangement such as is shown in Fig. 1 or, in more detail, in Fig. 2. This is the only place where rubber was permitted on the electrode side of the furnace; here it was amply protected by mercury.

The purity of the nitrogen prepared and delivered in this way was occasionally tested with alkaline pyrogallol in the apparatus

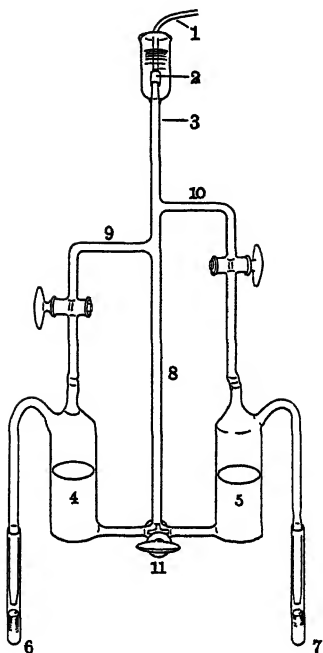


Fig. 2. Apparatus for testing the purity of nitrogen. Nitrogen is led through copper tubing (1) attached by means of short rubber tubing (2) to glass tubing (3); the rubber tubing is protected from air by a glass cup filled with mercury. Gas is then led *via* glass tube (8) through vessel (4), containing 10 per cent NaOH, after proper positions have been given stop-cock (11) and mercury valve (6). After NaOH is saturated with nitrogen, the gas is led *via* (8) into vessel (5) containing a pyrogallol solution acidified with a little acetic acid. Then with stop-cocks and mercury valves (6, 7) properly set, nitrogen is led over glass tube (9), and the liquid of (4) is pressed into (5) for mixing. In a similar way, the liquid is forced into vessel (4); after thorough mixing it is divided equally between (4) and (5). Vessel (5) is then permanently sealed by the 3-way stop-cock (11) and mercury valve (7) and gas is led *via* (8) through (4) to the outlet (6). Before the experiment is performed it is advisable to place a drop of dilute H_2SO_4 above stop-cock (11); this prevents premature appearance of color in case pyrogallol and alkali are mixed in spaces of the stop-cock.

Any trace of oxygen in nitrogen is shown by difference in color of (4) and (5) after some minutes of bubbling.

sketched in Fig. 2. This apparatus permitted washing out of oxygen by nitrogen from solutions of acidified pyrogallol and NaOH before mixture of the two; permitted mixture under a nitrogen atmosphere; and provided a standard of comparison for the color changes to be looked for. An hour of vigorous bubbling with purified nitrogen failed to produce detectable color change in the alkaline pyrogallol; 5 minutes slow bubbling with 1 part of oxygen to 40,000 of nitrogen brought an appreciable color difference.

Electrode vessels of various forms were used. That of Fig. 1 was both convenient and trustworthy. All leads were sealed with mercury.²

A calomel electrode in 4 M KCl was used as leading off electrode. It was frequently standardized against a hydrogen electrode in standard acetate buffer, the pH of which was taken equal to 4.62 in the temperature range between 18–38°.

All measurements were made in a large air thermostat with electrically controlled thermoregulator and a strong air fan. Most measurements were at 38°.

Potentials were read with nitrogen bubbling slowly through the electrode vessel. This precaution helps guard against accumulation of oxygen from the one unavoidable source of diffusion, the agar bridge connecting the electrode vessel to the calomel electrode. Moreover the nitrogen was bubbled at a definite rate. The amount of agitation of the cysteine solution is of great importance in measurement of potentials under known oxygen pressures; though insignificant in its effect in a pure nitrogen atmosphere, this safeguard was taken in the interest of complete control of experimental conditions.

In order to regulate the amount of agitation, it was first of all necessary to provide means of measuring and controlling the nitrogen flow. This was accomplished by use of a calibrated flowmeter of a type shown in Fig. 3 (one line of level difference in the arbitrary scale meant a flow of 1.56 cc. of N₂ per minute; the ratio of level difference and flow was constant over the employed range). Other obvious experimental conditions were then made such as to insure reasonable regularity in bubble size from experi-

² We are indebted to Mrs. Max Oates for the drawings in this communication.

ment to experiment. The absolute flow as indicated by the flowmeter could, therefore, be taken as a measure of the amount of agitation of the liquid.

A special series of experiments necessitated control of oxygen pressure. This was effected by a second flowmeter; oxygen either from an air tank or from a tank of analyzed, unpurified nitrogen, passed through it and so was measured, then, mixed with a measured quantity of purified nitrogen, was introduced into the copper tubing leading to the electrode vessel. In this way it was

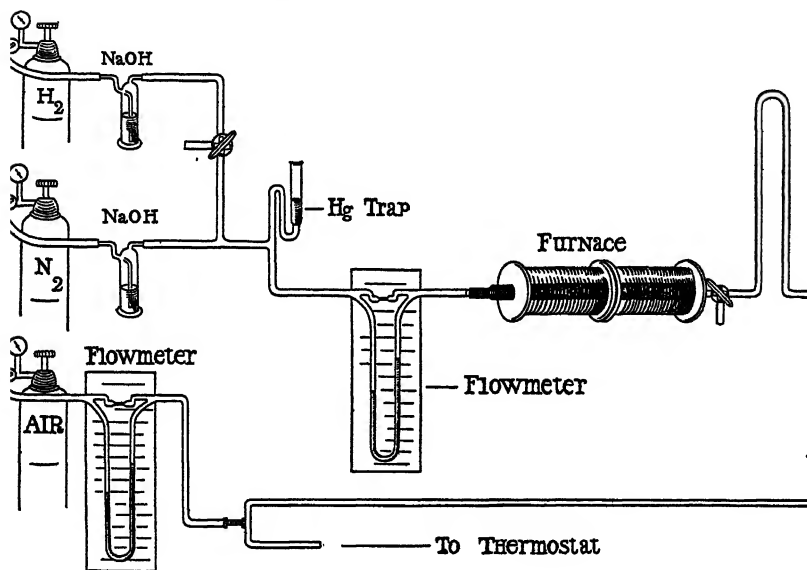


FIG. 3. Scheme of the gas leads.

possible to vary the ratio of oxygen to nitrogen from 1:40,000 to that of air. The device was entirely satisfactory from point of view of facility of operation and constancy of the gas pressures it supplied.

A tank of hydrogen was permanently joined by way of a 3-way stop-cock to the copper tubing entering the combustion chamber. This gas, freed of CO_2 by NaOH, was, when occasionally needed, passed through the oven at a temperature of $550-600^\circ$ to rid it of oxygen.

The cystine used was the commercial preparation of the Eastman Kodak Company. Cysteine hydrochloride was prepared from it by reduction with tin and HCl according to Baumann (1883-84); the final purification was made with acetone as suggested by Warburg.

The preparation was found to contain the theoretical amount of HCl by titration with NaOH, methyl orange being used as indicator. It also consumed the theoretical amount of iodine when, following Warburg (1927), titration was conducted in solutions of 95 per cent alcohol.

The potentiometry itself required no notable deviation from the standard methods. A Leeds and Northrup potentiometer type L and a galvanometer with telescope reading were used.

We are indebted to Dr. W. M. Clark for many personal suggestions used in development of the applied technique for purification of nitrogen.

Cysteine Potential at Blank Platinum Electrodes.

A long time is required for the establishment of the final, definite potential of a cysteine solution at a bright platinum electrode. A rather negative potential is established even in the presence of air; when the air is bubbled out with nitrogen, however, the potential becomes more and more negative. This shift is quite rapid in the beginning, then becomes progressively slower, and finally so slow that after the apparent end of an experiment 1 or 2 more hours are necessary for certainty that the final value has been reached. This is true both for temperatures of 22° and 38°. It is, therefore, necessary to make observations over a period of from 4 to 6 hours, depending on the electrode, before one is justified in calling a potential reading the ultimate one.

There are apparently two reasons for this slow establishment of the potential. As is to be shown in detail later on, the blank platinum electrode is extremely sensitive to even the smallest traces of oxygen; oxygen must, in consequence, be completely driven out of the solution. In agreement with this, if, after reaching the final potential, a trace of air was led into the electrode vessel, the potential was immediately shifted to the positive side. When bubbling with nitrogen was resumed, reestablishment of the end value took much less time than at the beginning of the experi-

ment; in fact, just about that time necessary for expulsion of the oxygen. This observation suggested that a change in the electrode itself is involved in the initial establishment of the potential. Recourse was had to the well known ideas concerning oxides or suboxides on the metal surface. From this point of view, the ultimate potential must await not only complete displacement of oxygen from solution but complete reduction of the surface layer of the metal as well. At the beginning of an experiment, the metal strips are all coated with oxides; it takes a long time for their complete reduction, and longer for the reduction of one strip than for another, for it is unlikely that the state of oxidation is identical for different samples. After complete reduction and establishment of the final potential, momentary introduction of a trace of air to shift the potential to the positive side produces little or no oxidation of the metal; the potential is quickly reestablished in consequence by bubbling with nitrogen.

Another experiment can be devised to demonstrate that the metal surface undergoes change on contact with the cysteine solution and reaches a stable state only after a long time. Two platinum electrodes were placed in the same vessel; one was immersed in, the other was left out of the solution. Oxygen was then bubbled out of solution with nitrogen and the bubbling continued until the immersed electrode reached its end value. The dry electrode was then pushed into the liquid; its potential was 100 to 200 millivolts positive to that of the first electrode, which remained unchanged during the manipulation. The positive potential changed after much time to the negative value established at the electrode which had been in solution from the beginning of the experiment. Several experiments verified these observations. It is certain, therefore, that contact of the electrode with the cysteine solution, freed of oxygen, induces a change necessary to the establishment of the final potential; this change, we repeat, very probably involves reduction of metastable oxides or suboxides of the platinum surface.

If the precautions growing out of the foregoing considerations were exercised, it was possible to reproduce at all platinum electrodes the potentials of definitive cysteine solutions to within 5 millivolts. Certain electrodes, of course, became unsatisfactory with time; this may have been due to minute cracks in the glass,

TABLE I.

Potential observed at 38°, in volts. Potential of hydrogen electrode in standard acetate pH 4.62 and 1 atmosphere of hydrogen pressure taken equal to zero.

pH	Concentration.	Blank platinum.	Gold-plated platinum.	Mercury.	Solid gold.	Potential calculated on basis $E_0 = +0.284$.
1.15	M/3100			+0.420		+0.426
2.04	M/100			+0.277*		
				+0.278*		+0.280
				+0.273**		
				+0.273**		
2.13	M/100			+0.272		+0.276
4.50	M/100	+0.141*	+0.145	+0.137*		
		+0.141*		+0.137*		+0.131
7.25	M/100	-0.038*	-0.031*	-0.039*		
		-0.037*	-0.035*	-0.040*		
		-0.038**	-0.030**			-0.036
		-0.038**	-0.032**			
8.8±0.1	M/100	-0.132*				
		-0.132*				
		-0.134	-0.135	-0.137		-0.130
9.5	M/2000			-0.081*		
				-0.079*		-0.098
12.46	M/100			-0.223		-0.363
4.62	M/3600			+0.213*		+0.219
				+0.213*		
4.62	M/3000	+0.218*				
		+0.218*				
		+0.218*				+0.215
		+0.217*				
4.62	M/1000	+0.187*				
		+0.184*				
		+0.185*				
		+0.189**				+0.183
		+0.189**				
7.30	M/1000		+0.041			+0.035
4.62	M/900			+0.182		
				+0.181		+0.182
4.60	M/300	+0.153*		+0.152*		
		+0.157*				+0.153
		+0.156**		+0.153*		
		+0.156**				
		+0.155**				
		+0.155**				

TABLE I—*Concluded.*

pH	Concentration.	Blank platinum.	Gold-plated platinum.	Mercury.	Solid gold.	Potential calculated on basis $E_0 = +0.284$.
4.53	M/100	+0.142 ^s +0.141 ^s +0.141 ^s +0.141 ^s +0.140 ^{*s} +0.141 ^{*s} +0.142 ^{**s} +0.139 ^{**s}		+0.137 ^s +0.137 ^s +0.130 ^{*s} +0.131 ^{*s}	+0.299 ^s +0.271 ^s +0.289 ^{*s} +0.317 ^{*s} +0.305 ^{**s} +0.080 ^{**s}	+0.128
3.40	M/10	+0.141 ^s +0.143 ^s				+0.136
4.46	M/6500			+0.230 ⁺¹		+0.224
4.76	M/3000			+0.205 ⁺¹		+0.205
4.76	M/1390			+0.187 ⁺¹		+0.185
4.76	M/1355			+0.179 ⁺²		+0.182
4.76	M/720			+0.170 ⁺¹		+0.168
4.76	M/702			+0.165 ⁺²		+0.167
4.76	M/360			+0.153 ⁺¹ +0.149 ⁺²		+0.149
4.76	M/244			+0.146 ⁺¹		+0.140
4.76	M/239			+0.139 ⁺²		+0.137

Values marked (s) are from experiments in which electrodes were in same vessel and so under exactly the same experimental conditions. Values marked (*s), from repetition of these experiments; those marked (**s) are from a third similar series. Values marked (+1) were obtained in one titration experiment; those marked (+2) came from another.

leading to establishment of secondary circuits or to other causes still unknown. Electrodes of irregular behavior, however, were not met with more often than in ordinary cases, *e.g.* in quinhydrone electrodes; moreover the faulty electrode was easily recognized from its behavior toward quinhydrone, and discarded. Reproducibility, in fact, was nearly as good as in any well established redox system. We worked for a long time with an electrode vessel containing six single platinum electrodes. One of these always showed a slight irregularity even in quinhydrone; the other five, which behaved regularly towards quinhydrone, all gave the same potential for cysteine though, as has been pointed out above,

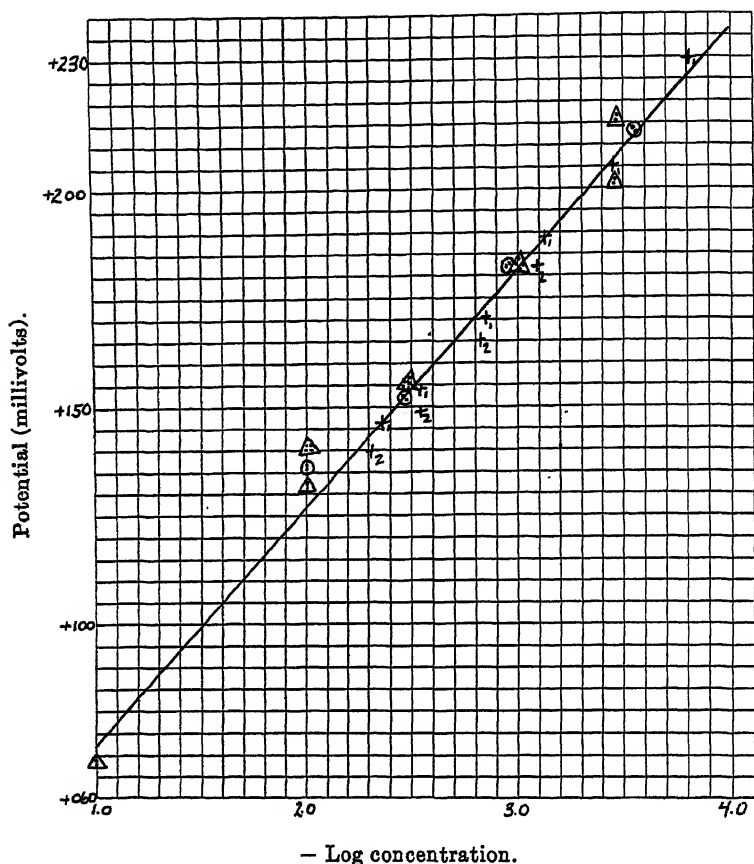


FIG. 4. Variation of potential with variation of concentration of cysteine. The line drawn is that fixed by formula: $E = + 0.284 - \frac{RT}{F} \log [\text{RSH}] + \frac{RT}{F} \log [\text{H}^+]$. Δ = values from individual experiments with blank platinum electrodes. \circ = values from individual experiments with mercury electrodes. $+_1, +_2$ = values from titration experiments with mercury electrodes. pH 4.62, temperature 38°. Potential of hydrogen electrode in standard acetate pH 4.62 and 1 atmosphere of hydrogen pressure taken equal to zero. To obtain values referred to normal hydrogen electrode subtract 285 millivolts. Each point is defined by an experiment.

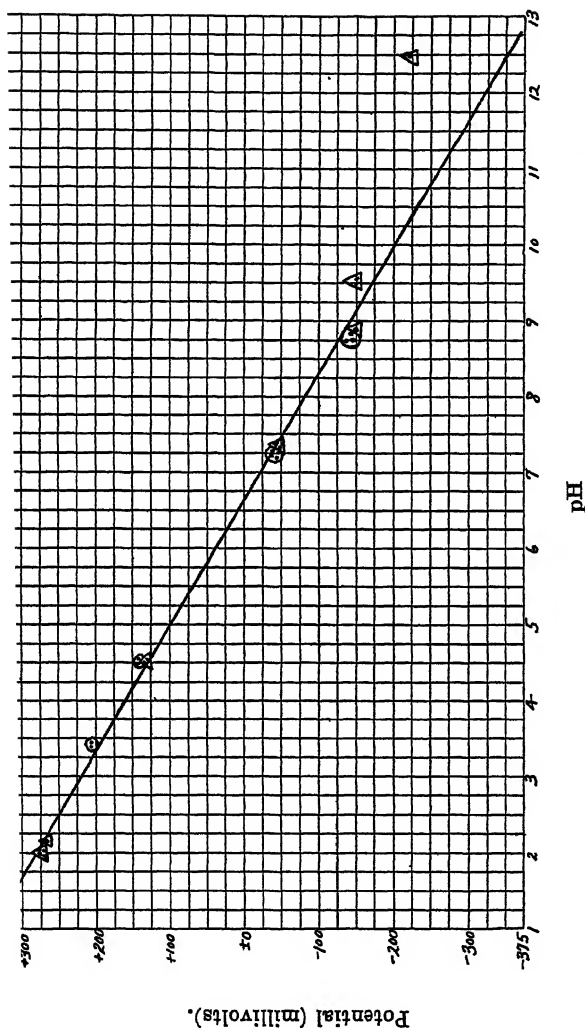


Fig. 5. Variation of potential with variation of pH. The line drawn is that fixed by formula: $E = +0.284 - \frac{RT}{F} \log [RSH] + \frac{RT}{F} \log [H^+]$. Δ = values from experiments with mercury electrodes. \circ = values from experiments with blank platinum electrodes. Concentration of cysteine = 0.01 M; temperature 38°. Potential of hydrogen electrode in standard acetate pH 4.02 and 1 atmosphere of hydrogen pressure taken equal to zero. To obtain values referred to normal hydrogen electrode subtract 285 millivolts. Each point is defined by an experiment.

different lengths of time were necessary for attainment of the final potential with different electrodes.

A. Variation of Potential with Variation in Cysteine Concentration.—The results in Table I and Fig. 4 show that at the pH 4.6 the potential is a logarithmic function of the concentration of cysteine. Within a rather large range of concentration, from 0.1 to 0.0002 M, an increase of the concentration by 10 times makes

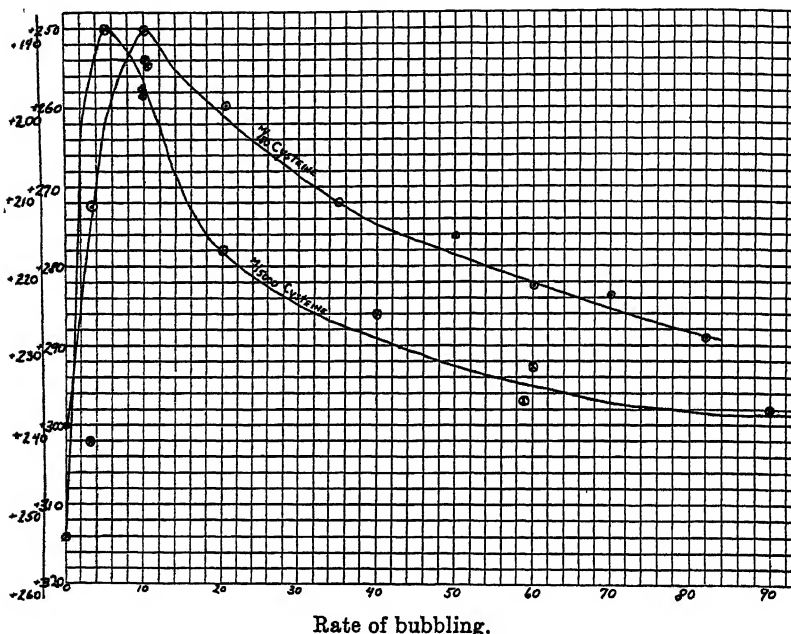


Fig. 6. Variation in potential with variation in rate of bubbling. Nitrogen atmosphere with 0.08 per cent oxygen; temperature 38°. ○ = values for 0.02 M cysteine at pH 4.5. ⊗ = values for 0.0002 M cysteine at pH 4.6. The left-hand ordinate scale holds for ○, the right-hand ordinate scale for ⊗.

the potential at 38° more negative by 0.061 volt. All the results were obtained by individual experiments for each concentration, not by titration.

B. Influence of pH.—Table I and Fig. 5 show that within the limits of reproducibility described before, the potential depends in a logarithmic way on the hydrogen ion concentration. Each change of a unit of pH at 38° produces a change of 0.061 volt.

This was observed over a range of pH from 1.2 to 9.4. The pH of the solution *in toto* was directly measured in each experiment. Hydrogen electrode values with freshly platinized platinum electrodes were checked by the Sørensen method with Clark indicators and by the method of Michaelis and Gyemant with the nitrophenol series.

C. Influence of Oxygen Tension.—The behavior of the cysteine potential in presence of oxygen shows three striking characteristics. (1) The presence of oxygen makes the potential more positive; there is an increase in positiveness with increase in oxygen tension. (2) The establishment of a definite potential in presence of oxygen requires much less time than with purified nitrogen. (3) In the presence of a small amount of oxygen, the potential depends to a remarkable degree on the speed of gas bubbling through the solution or on the amount of stirring or shaking of the solution. This is especially the case for mixtures of nitrogen with very small amounts of oxygen (over the range of 1 part of oxygen to 40,000 of nitrogen, to 1 part of oxygen to 100 parts of nitrogen), whereas this shaking effect is little noticed in high oxygen tensions, such as that of air, and is insignificant in purified nitrogen.

The influence of variation in rate of bubbling is shown in Fig. 6. In these experiments the solution was saturated with gas from a tank of unpurified nitrogen; the ratio of oxygen to nitrogen was 1:1250. A difference of 70 millivolts was produced by variation in rate of bubbling. There was a maximum of negativity of the potential at a rather slow rate of bubbling; an extremely sharp decrease of negativity with further decrease in rate of bubbling; a much slower decrease, tending to an asymptotic value, with increase in the bubbling rate.

The effect of variation of oxygen pressure is shown in Fig. 7 and Table II. Such an experiment to be performed rigidly must take account of the agitation of the solution. This condition was not strictly controlled; it was regulated quite nicely, however; certainly closely enough to interfere in no important way with the results. The curve is selfexplanatory; even the slightest trace of oxygen pushed the potential to the positive side. An amount of oxygen amounting to 0.003 volumes per cent caused a decrease in negativity of 15 millivolts. The oxygen content of the solution is thus seen to be of enormous importance in determination of the potential.

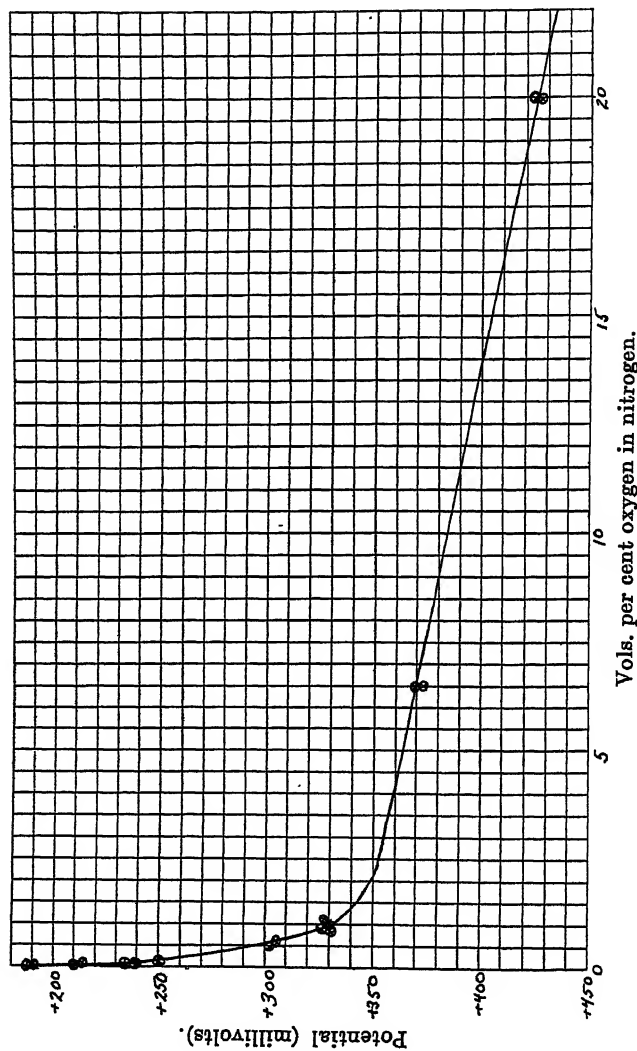


Fig. 7. Variation of potential at blank platinum electrode with variation of oxygen tension. 0.0002 M cysteine at pH 4.62, temperature 38°. Potential of hydrogen electrode in standard acetate pH 4.62 and 1 atmosphere of hydrogen pressure taken equal to zero.

D. Influence of Hydrogen on Potential.—There is an increase in negativity of the potential if the solution is bubbled with pure hydrogen after establishment of the final potential in presence of nitrogen. The value of this potential is always more positive than the hydrogen potential with platinized platinum; with practically all blank electrodes it is some 200 millivolts more positive. One receives the impression that bright platinum has

TABLE II (*Data of Fig. 7*).

Variation of Potential of Platinum Electrode with Variation of Oxygen Tension.

0.0002 M cysteine at pH 4.62; temperature 38°. Potential of hydrogen electrode in standard acetate pH 4.62 and 1 atmosphere of hydrogen pressure taken equal to zero.

Vol. per cent of O ₂ added to purified N ₂ .	Potential.
	<i>volts</i>
0.000	+0.190
0.003	+0.205
0.1	+0.250
0.6	+0.308
1.0	+0.330
	+0.328
6.5	+0.370
	+0.374
20.0	+0.425
	+0.430
1.0	+0.325
	+0.332
0.5	+0.302
0.01	+0.235
0.02	+0.240
0.004	+0.210
0.000	+0.188

the tendency to work as a true hydrogen electrode; *i.e.*, that it attempts establishment of thermodynamic equilibrium between hydrogen gas and hydrogen ions but that the velocity of this process is very slow and the process itself incomplete. The relatively slight effect of hydrogen can be entirely dissipated by renewed bubbling with nitrogen. The presence of cysteine, then, in a buffer solution in no way alters the typical behavior of blank platinum as a hydrogen electrode in pure buffer solutions.

E. Effect of Polarization.—Every care must be taken to avoid polarization in reading the potentials; even weak polarization brings about a change of some tenths or a whole of 1 millivolt. The displaced potential, however, rapidly returns to its normal value. This holds not only for the final value which is reached after hours of bubbling but in addition for those measurements taken during the course of the drift. The drift, it is to be recalled, is quite gradual; the potential returns so promptly and completely from momentary polarization that the disturbance alters its course in no appreciable way.

If the final, definite potential be forced up or down by say 200 millivolts on extended polarization, it returns spontaneously and exactly to its original value after polarization; this is an important fact for understanding the significance of the final potential. The velocity at which the potential returns varies greatly with the direction of polarization. After cathodic polarization, there is a rapid return to the true potential; after anodic polarization, the shift is slow and drifting.

These observations indicate that the cysteine potential is that of a system at equilibrium. Its full significance is lost, however, in our ignorance of the mechanism of establishment of the potential; their explanation must, in consequence, be postponed.

Cysteine Potential at Gold Electrodes.

The detailed description just given of the behavior of blank platinum electrodes fits quite well observations made with gold-plated platinum. There are only two differences: gold responds even less to hydrogen than does platinum and reaches its final potential even more slowly. The ultimate value of the cysteine potential in pure nitrogen is, within the limits of reproducibility, the same in the two sets of electrodes. Individual differences in the behavior of various gold-plated electrodes appear only in variation of the time necessary for establishment of the definite potential.

The behavior of electrodes of pure, solid gold is very different. A constant, final potential is more quickly established than in blank platinum or gold-plated platinum. There is even less variation in the potential with change in atmosphere from nitrogen to hydrogen than in the gold-plated electrode. The major differ-

ence, however, and the difficulty which presents itself in the case of solid gold strips, is that the end potential is altogether different for each gold electrode and never so negative as in the other electrodes.

The electrodes behave as though their surfaces contain oxygen compounds scarcely reducible by cysteine. The potential, following this view, corresponds to that of a platinum electrode incompletely reduced. Different gold strips are oxidized to different degrees; to each degree of oxidation there is correlated a definite potential. This peculiarity in the case of a particular electrode does not exclude a variation in potential with change in cysteine concentration and change in pH according to the formula of Dixon and Quastel. It does, however, make acceptance of the potential as an intrinsic property of the solution itself impossible and for this important reason we have discarded solid gold electrodes for measurement of the cysteine potential.

Cysteine Potential at Mercury Electrodes.

A resting surface of pure mercury was used as electrode; there was no call for a device permitting renewal of the surface. The essential result is that the final potential agrees nicely with that at blank platinum or gold-plated platinum. There are definite differences in behavior, however. The end potential at mercury is much more quickly established than at other electrodes; the electrode is far less sensitive to oxygen; the potential is unaffected to a detectable degree by a change from nitrogen to hydrogen.

The rapidity with which the ultimate potential is reached at mercury is quite surprising (see Fig. 8). Even in air the potential is much more negative than at platinum; the negativity very rapidly increases when the air is bubbled out with nitrogen and comes to within some 10 millivolts of its ultimate value in 15 minutes or so. The last few millivolts appear quite slowly, requiring a half to a whole hour, sometimes longer. All in all, however, the mercury electrode yields the final potential so much more quickly than platinum that the equivalent of a week's work with the latter can be done in a day with the former.

The effect of oxygen on a mercury electrode is similar to that on a platinum electrode in so far as the gas always renders the potential positive to that in purified nitrogen or hydrogen. The

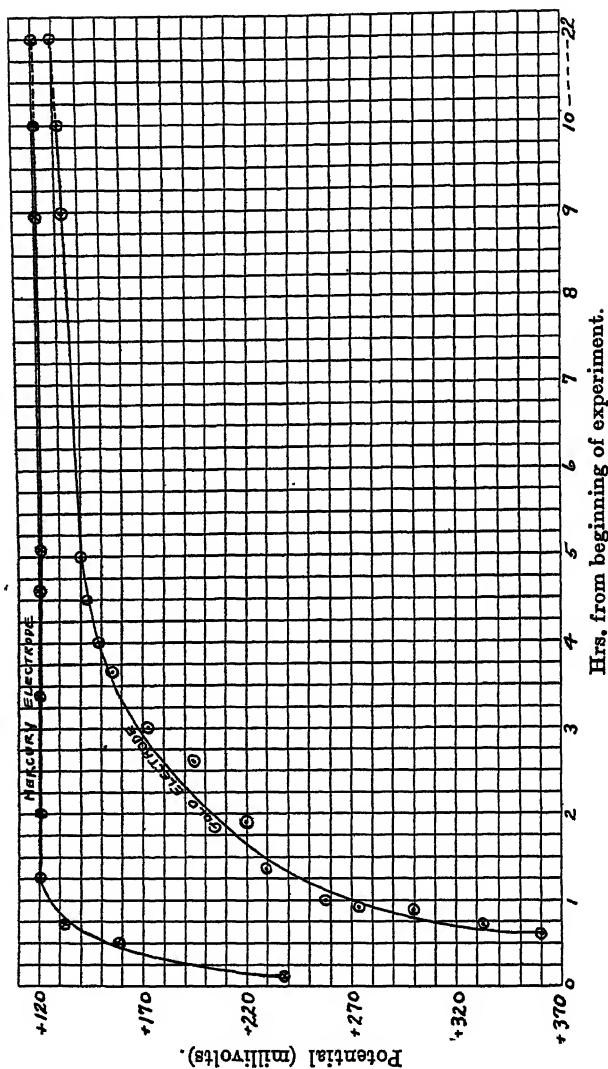


Fig. 8. Potential establishment at electrodes of mercury and gold-plated platinum in cysteine of pH 4.6 and purified nitrogen atmosphere. \circ = values for gold-plated platinum. \otimes = values for mercury. Temperature 38°. Potentials referred to the normal H_2 electrode.

magnitude of the influence, however, is much smaller. A change from a purified nitrogen atmosphere to an atmosphere containing 0.45 volume per cent of oxygen made the potential more positive by 12 millivolts in a 0.0002 M solution of cysteine, and more positive by 15 millivolts in a 0.01 M solution at pH 4.6 in standard acetate and at 38°. Rate of bubbling at low oxygen pressures is of much less consequence than in the case of platinum. It becomes very conspicuous, however, in oxygen pressures of the order of that of air. When, for example, a 0.0002 M cysteine solution at pH 4.6 was saturated with air, its potential at mercury was 180 millivolts more positive at rest than on moderate bubbling. Interpretation of this observation is not difficult; in presence of oxygen, traces of mercury go into solution as Hg^+ ions; a stationary condition is established when the rate at which Hg^+ ions are furnished into the solution is compensated by the rate at which they are reduced by cysteine. On bubbling, the Hg^+ ions are distributed throughout the entire liquid. At rest, they accumulate at the mercury surface. This explanation seems sufficient to account for the influence of bubbling on the potential at the mercury electrode in presence of oxygen. More detailed study of the phenomenon is thought beyond the scope of the immediate investigation.

The variation of the potential with concentration of cysteine and pH is to be seen in Table I and in Figs. 4 and 5. It is to be noted in accordance with an observation of Dixon and Quastel, that there is a deviation from the calculated potential at high pH. In our experiments this is first seen at a pH of 9.5; it is striking at pH 12.4. This deviation is apparently related to dissociation at the SH^- group; its full discussion is reserved for a later communication. The quick establishment of the potential at mercury permitted titration experiments in which the concentration of cysteine was varied by addition to the electrode vessel from a pipette. The results of these titrations are to be seen in Table I and in Fig. 4 where the values are noted by $+_1$ and $+_2$. The two titration experiments noted agree not only with each other but also with experiments singly performed at mercury and blank platinum.

Cysteine Potential at Platinized Platinum Electrodes.

The drift to the end potential at the platinized platinum electrode is even slower than at the bright metal; after as long as 8 or 9 hours one is not certain that the measured potential is the ultimate one. An effort was made to check that value got on bubbling out air with nitrogen by establishing a hydrogen potential and then bubbling out the hydrogen with nitrogen. If the potential considered final be truly final, there should be no discrepancy between the value in a nitrogen atmosphere, be it derived from the air or from the hydrogen side. The minimal difference between the two values in six sets of experiments was 3 millivolts; the maximal, 25 millivolts; the mean, 12 millivolts.

Beyond these differences, evident after most painstaking precaution in experimental detail, many platinized electrodes in the same vessel and so under exactly equivalent conditions show potentials which vary widely. Little exact, in consequence, can be expected from the results; we discarded the platinized electrode after much unsuccessful labor to control it for the same reasons which led to abandonment of solid gold. Our thought that the black electrode might serve better to measure the reduction potential of cysteine than it does to measure the redox potential of systems such as quinhydrone is, therefore, without foundation.

Influence of Cystine on Potential.

Dixon and Quastel, working with solid gold electrodes, have stated that the oxidation product of cysteine, cystine, is without effect on the cysteine potential. Our results wholly confirm this finding. We made many attempts to test this statement; we present the following experiments as typical examples.

1. A solution of 0.0002 M cysteine hydrochloride in standard acetate buffer pH 4.62 at 38° was placed in the electrode vessel containing blank platinum electrodes. At this acid pH, even in presence of iron, oxidation of cysteine before complete removal of oxygen by nitrogen can be neglected. When the final potential was reached, the vessel was opened for a moment; an excess of cystine crystals poured in, the vessel was closed, and bubbling with nitrogen resumed. On the basis of Sano's (1926) figures for the solubility of cystine at 25° at pH 4.67, the solution is finally 0.00042 M with respect to cystine; actually cystine's molarity is

greater since the experiment is at 38° . Even on supposition that one-quarter of the original cysteine is impurity of cystine, certainly a high exaggeration, the concentration of cystine is changed 10 times by addition of the crystals. The ultimate potential, nevertheless, was not affected to a detectable degree by the addition of cystine.

2. Even more convincing is the following experiment in which arrangements permitted a much wider variation of concentration of cystine. The potential of a 0.0003 M solution of cysteine in 0.1 M HCl was measured at a mercury electrode; then a parallel experiment made with cysteine of the same concentration (0.0003 M) in 0.1 M HCl saturated at 38° with cystine by shaking with a great excess of finely ground cystine crystals. The solubility of cystine in 0.1 M HCl is, according to Sano, almost exactly 30 times greater than in standard acetate and therefore more than 30 times greater than the cysteine concentration of the experiment. No matter what the impurity of cystine in our cysteine, the concentration of the former was highly increased; still the potential remained within the limits of change brought by pH differences.

3. An electrode vessel with a mercury electrode was filled with borate buffer at pH 9.4. Through the rubber stopper of the vessel went a glass rod on the end of which was fused a glass spoon. The spoon was filled with 10 mg. of cystine and kept above the solution during the first part of the experiment. The buffer solution was thoroughly bubbled out with nitrogen; then the vessel opened and enough of a 0.02 M solution of cysteine hydrochloride added to give a 0.0005 M solution of cysteine. The vessel was promptly closed and the potential followed to its final value. Cystine was then added by pushing the spoon into the liquid. The amino acid readily dissolved but again failed to affect the potential. The quantity of cystine used was small enough not to alter the pH appreciably but large enough to change the cystine concentration to 0.002 M from that very low concentration due to impurity in the 0.0005 M solution of cysteine.

Influence of Iron and of Cyanide on Cysteine Potential.

It is known in large part from the investigations of Mathews and Walker (1909) and Warburg and Sakuma (1923, 1927), that heavy metals, especially iron, play a great part in the

physiological rôle of cysteine and that cysteine forms a complex both with ferric and ferrous iron. The deep violet ferric complex is unstable and undergoes a rapid change into ferrous iron and cystine. It is of no importance, therefore, in establishment of the potential. But the ferrous complex of cysteine may be of significance. The presence of a trace of iron could not be excluded under our experimental conditions and one may be inclined to attribute an important function to the metal in accord with Warburg's observations that even slight traces of it permit consumption of molecular oxygen by cysteine.

Ferrous sulfate added to give a concentration of 0.001 M to a 0.01 M solution of cysteine at pH 4.6 in standard acetate buffer was, however, without effect on the potential. The iron content of pure cysteine in pure buffer though not zero was negligible compared to the iron content of the above solution as shown by comparative thiocyanate reaction. Moreover, KCN added to a concentration of 0.01 M at pH 7.4 to a solution containing no more iron than its accidental traces effected no change in the potential. Variation of the iron content of the solution between extremely wide limits must, in consequence, be considered without effect on the potential established by the free cysteine. Obviously the amount of iron is concerned with the velocity of consumption of molecular oxygen by cysteine, and, according to S. Toda (in Warburg's laboratory (1928)), also in the rate of reduction of methylene blue by cysteine, but an extreme variation in its concentration has nothing to do with the magnitude of the potential established at an electrode in absence of oxygen.

DISCUSSION.

A. Physicochemical.

The potential of the cysteine system, to be in accord with the principles of reversible redox systems, should be a function of the logarithm of the cysteine concentration and of the logarithm of the reciprocal value of the square root of cystine's concentration. A function of these dimensions can be expected even though there be intermediary steps in the oxidation of cysteine to cystine. Experience shows, however, that the potential is completely independent of the concentration of cystine.

In our present knowledge we attempt explanation of this fact by assuming the process of potential establishment to be irreversible. This means that cysteine charges the electrode with hydrogen atoms³ but that cystine fails to withdraw hydrogen atoms from the metal. In so far as cysteine's tendency to charge the metal with hydrogen is not counterbalanced by a reverse reaction, the potential can be expected to reach an infinitely high negative value; or, since the potential in an aqueous solution cannot exceed a hydrogen potential (except for an overvoltage which is always finite), the potential should be expected to be at least that of a hydrogen electrode. This, obviously, is false.

Dixon, attempting an explanation, assumes that charge of the metal with hydrogen by cysteine is counterbalanced by diffusion of hydrogen out of the metal into the solution. It is unbelievable, however, that atomic hydrogen diffuses into the solution to be maintained in this state. If hydrogen does diffuse into the solution, it can be present there only in the form of dissolved hydrogen molecules. And if blank platinum and mercury, charged with hydrogen atoms, have the faculty of developing hydrogen molecules and so of establishing equilibrium between hydrogen molecules in solution and hydrogen atoms in the metal, then diffusion from the metal should be stopped by saturating the solution with hydrogen gas. A cysteine solution bubbled with hydrogen should, in consequence, establish a hydrogen potential. Cysteine solutions at electrode of blank platinum on being bubbled with hydrogen never reach the hydrogen potential; and at mercury there is not even a detectable difference in the potential, be the solution bubbled with hydrogen or nitrogen.

A more acceptable explanation in the light of this criticism would involve a diffusion of hydrogen atoms from the surface of the metal, not into the solution, but into the body of the metal itself. The capacity of metals, taking into consideration their entire bulk, is high for hydrogen atoms. This explanation, however, has to be discarded with the first for a continuous supply of hydrogen atoms to the electrode must result in progressive oxidation

³ It is inconsequential whether we consider electrons from the molecule ionized at the SH- group or hydrogen atoms from the undissociated SH-group to leave cysteine and charge the electrode. For this reason it may suffice to say that cysteine charges the electrode with hydrogen.

of the cysteine, progressive diminution of its concentration, and a consequent drift of the potential to the positive side. According to our experience, however, the cysteine potential even in very dilute solutions is constant for as long as 24 hours. Moreover, it is highly improbable that the velocity of migration of atoms and protons is the same within platinum, gold, and mercury and so incompatible with the fact that the potentials at the three metals are equivalent. In consideration of these objections, diffusion of hydrogen atoms from the surface into the body of the metal must be negligible compared to the rate of supply of hydrogen atoms by cysteine.

In experimental support of his theory, Dixon pointed out that the magnitude of the cysteine potential depends on the nature of the "indifferent" electrode. He found the potential about 200 millivolts more negative at mercury than at solid gold; this observation was in accord with the high hydrogen overvoltage of mercury and in agreement with his theory.⁴ As our experiments show, however, the magnitude of overvoltage of various metals can in no way be correlated with the potentials established at them. The two metals, blank platinum and mercury, which are, when we disregard platinized platinum, the two limiting members of the overvoltage series of metals, give accurately the same potential in cysteine solutions and differ only in the speed with which the potential is established. It was probably accidental that Dixon compared mercury with solid gold; solid gold is, according to our experience, an exceptional and unacceptable case.

The shift of the potential in presence of oxygen and the variation in sensitiveness to the gas on the part of different metals can be accounted for in the following two ways. All of the noble metals have a tendency to behave as oxygen electrodes; that metal which is most efficient in dissociating molecular oxygen into atoms

⁴ We have adopted that theory of cathodic polarization and hydrogen overvoltage which states that hydrogen deposited by an electric current at the cathode is deposited in the atomic state and is loosely combined with the metal atoms. The theory makes the further assumption that transformation of atomic hydrogen into molecular hydrogen is dependent upon catalysis by the metal. Differences in catalytic capacity impart different overvoltages to different metals. (A summary of the theories of overvoltage is given by Baars, E., in *Handbuch der Physik*, Berlin, 1928, xiii.)

and of establishing equilibrium between the two will form the best oxygen electrode and will be most sensitive for oxygen. It is known from wide experience that platinized platinum, though far from ideal, is best fitted to these ends; next comes blank platinum, then gold.⁵ Our experiments with cysteine show a sensitivity of the potential to oxygen at these metals of exactly this order. The results can also be accounted for in another way, basically dependent on those properties of the metals just discussed. Oxygen oxidizes the hydrogen sticking in the electrode surface. The velocity of this process depends upon the catalytic property of the particular metal and therefore upon its capacity for oxygen and hydrogen and upon the facility with which it disrupts the molecules of gas into atoms. This catalysis is extremely poor at mercury. It is higher in blank platinum. In platinized platinum it is so great that permanent charge of the electrode with that amount of hydrogen corresponding to the reducing power of cysteine is almost impossible; the hydrogen, as it is supplied by cysteine, is eaten away from the electrode even by the smallest trace of oxygen.

In presence of a constant oxygen pressure there is a dynamic process at a rather constant level. Conditions, however, are somewhat unstable; change of degree of agitation of the liquid produces marked differences in potential. In a purified nitrogen atmosphere, on the other hand, the whole aspect of the potential as judged by constancy and reproducibility of its final definitive value, complete recovery from anodic and cathodic polarization, freedom from disturbance by changes in agitation, is that of the potential of a system in true equilibrium.

Objection may be made against considering the potential that of a true equilibrium rather than that of a dynamic process at a constant level in Dixon's sense. It is impossible to decide whether

⁵ Nothing can be said of mercury in this respect. Mercury acts as an indifferent electrode only in the presence of strong reducing agents which reduce mercurous ions to that level where the potential taken as a mercury electrode potential is equal to the potential of the redox system itself, mercury being considered as an indifferent electrode. The effect of oxygen at a mercury electrode may be accounted for by a production of mercurous ions above the level of equilibrium with the redox system or by a consumption of atomic hydrogen furnished to the electrode by the reductant.

the nitrogen used in our experiments was absolutely oxygen-free in the most rigid sense. We have shown that in presence of oxygen the potential is determined by the partial pressure of this gas. One may be inclined to the assumption, in consequence, that the anaerobic potential called by us final was really controlled by traces of oxygen too slight to be detectable. The following facts may refute such an assumption.

1. It has been shown that the change of potential at platinum with change in oxygen pressure is particularly steep in the range of the very lowest oxygen pressures. It will be remembered that the potentials established in presence of purified nitrogen were well reproducible at all times; in order to account for this in the light of the objection raised we would be forced to the assumption that the quantity of oxygen in our purified nitrogen was always the same. It is unreasonable to believe, however, that there was always accurately the same trace of oxygen present in purified nitrogen. This condition would have necessitated stricter constancy of temperature of the oven and constancy of the copper surface over which the nitrogen was led than was sought in our experiments. Moreover, it is unlikely that possible traces of oxygen would accompany purified nitrogen and purified hydrogen in accurately the same quantity; it will be remembered, however, that the potential at mercury was not to be varied by change from a nitrogen to a hydrogen atmosphere.

2. The potential at any definite oxygen pressure is very different at platinum than at mercury. We found, however, the same potential at platinum and at mercury in an atmosphere of purified nitrogen. We are forced to the conclusion again that our nitrogen contained either no oxygen at all or traces too slight to affect the potential in any way. So we feel justified, therefore, in considering the cysteine potential as that of a system in true equilibrium though we are unable to state that chemical reaction which is kept at equilibrium.

We have indicated that we think Dixon's theories impossible to accept. We are at a loss, however, to offer in substitution a comprehensive explanation of the peculiar behavior of cysteine. Elucidation of the observations and problems we have spoken of must await, therefore, further study and experimentation.

B. Physiological.

We return now to the problem set forth in the introduction: In absence of oxygen, are cysteine or other sulfhydryl derivatives indicators for what we can call the reduction power of the whole tissue system as is the methylene blue-methylene white system? In attempting to answer this question we are faced with an entirely new situation. Cysteine or its derivatives, natural constituents of the tissue liquids, certainly determine a definite potential in absence of oxygen. But, whereas in a system such as methylene blue the mechanism of potential establishment is clear, the potential of the sulfhydryl bodies is inexplicable. We can with good reason say, however, that the sulfhydryl bodies are present in high enough concentration to poise the tissue and to account for the reduction of all indicators by living cells recently demonstrated by Cohen, Chambers, and Reznikoff (1928). It is wholly likely that they impress their own level of reduction power upon those other tissue systems, the state of which can be expressed in terms of potentials.

It should be kept in mind that the sulfhydryl bodies play another important rôle in the organism dependent upon their activation of oxygen in presence of traces of heavy metal salts, as has been shown by Warburg. It is too early to build a hypothesis as to how oxygen activation and potential establishment are interwoven. Full explanation must await an understanding of the mechanism of the electrode potential.

SUMMARY.

A solution of cysteine in absence of oxygen establishes a definite potential at an indifferent electrode. This potential is independent of the concentration of cystine and depends logarithmically on the concentration of cysteine and of hydrogen ions. The potential is identical at electrodes of blank platinum, gold-plated platinum, and mercury, and reproducible to within 5 millivolts. Slight traces of oxygen displace the potential to the positive side. Mercury is much less sensitive to oxygen than platinum or gold-plated platinum and has the great advantage of yielding final values in a relatively short time. The anaerobic potential is negative enough to account for reduction of all indicator dyes by

cells under anaerobic conditions. The potential E , referred to the normal hydrogen electrode and measured in absence of oxygen, can be expressed in volts by the formula:

$$E = -0.001 - \frac{RT}{F} \ln [\text{cysteine}] + \frac{RT}{F} \ln [\text{H}^+]$$

No satisfactory theory can be offered for the mechanism by which this sharply fixed potential is established by cysteine which, in presence of its oxidation product, cystine, fails to form a truly reversible system.

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FURTHER EVIDENCE OF THE COMPLEX NATURE OF VITAMIN B.

I. EVIDENCE THAT A THIRD FACTOR EXISTS.*

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The vitamin originally known as vitamin B has been definitely shown to be made up of at least two separate and distinct vitamins. Reference to the literature concerning the dual nature of vitamin B has recently been given by Sherman and Axtmayer (1) and in a recent article (2) from this laboratory. This laboratory has modified a well known method for the purpose of separating these vitamins from each other in order that investigations may be carried out as to their exact properties.

Method.

The method of Seidell (3, 4) has been followed in a general way. 1600 gm. of fresh dry yeast were suspended in 12 liters of 0.01 per cent acetic acid and allowed to autolyze with occasional stirring, for 24 hours. The autolyzed material was centrifuged and the supernatant liquid poured off. The solids were shaken up twice with 0.01 per cent acetic acid and centrifuged, and the washings were combined with the original liquid portion. The liquid portion was then concentrated to 5 or 6 liters before an electric fan and then treated with fuller's earth in the proportion of 100 gm. of earth to 1000 cc. of liquid. This was stirred for 4 or 5 hours with an electric stirrer and then filtered on a Buchner funnel with suction. The earth was washed three times with distilled water. The filtrate was treated in the same way the second time and then

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concentrated before an electric fan to a volume of 1600 cc. This fraction as will be shown later contains the vitamin that prevents pellagra-like symptoms in rats and which with the other part of the vitamin B complex induces growth. This hereafter will be designated as the B fraction or antipellagra vitamin (vitamin G).

The other fraction of the vitamin B complex was removed from the moist fuller's earth by means of a concentrated solution of barium hydroxide. The liquid was separated from the earth by filtering and washing with suction on a Buchner funnel. The filtrate was neutralized immediately with sulfuric acid, litmus paper being used as an indicator. The earth was extracted the second time and the filtrate neutralized as before. The barium sulfate was centrifuged off and the liquid concentrated before an electric fan to 1600 cc. This hereafter will be designated as the A fraction and contains the vitamin that prevents polyneuritis, and which with the B fraction produces growth in rats. It is also designated as the antineuritic vitamin, or vitamin F. This terminology is in agreement with that of Sherman and Axtmayer (1) and is one which seems logical.

The data reported in this paper include: (1) A study of the effect of each of these fractions (vitamins) separately and in combination on the growth of rats, and (2) A study of the effect of each of the separate fractions (vitamins) as supplements to other diets. Several check or control groups are included.

EXPERIMENTAL.

The rats used as the experimental animals were 24 days old and weighed from 40 to 70 gm. when placed on test. They were kept individually in screen bottom cages and fed on a basal vitamin B-free diet consisting of casein 18; starch 64; salt mixture¹ 4; Crisco 10; agar agar 2; and cod liver oil 2. The vitamin preparations were fed daily and separately. From an inspection of the growth curves (Chart 1) it is seen that the fractions A and B, or vitamins F and G (Sherman's terminology), when fed separately, stimulate slight growth, or extend the maintenance period over that of the basal diet, but loss in weight soon follows. Those on small amounts, 0.6 cc. or less, of the B fraction invariably died of polyneuritis.

¹McCollum's Salt Mixture 185. (McCollum, E. V., and Simmonds N., *J. Biol. Chem.*, 1918, xxxiii, 63.)

Such were not the results when 1.0 cc. or more was used and it is not certain from this whether these fractions are pure, or whether

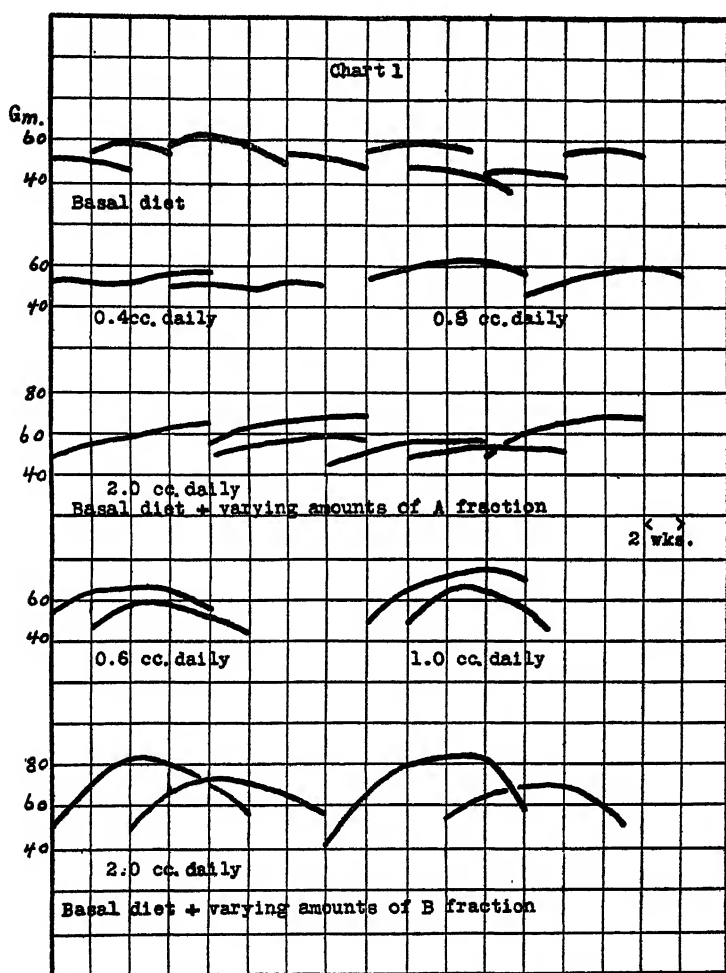


CHART 1.

they supplement the vitamins that are already stored in the body and thus the animals are able to maintain their weight for a longer time. When these two fractions are fed in combination (Chart 2)

there is continuous growth, but the growth does not compare favorably with that produced by an equivalent amount of the original yeast (1.0 cc. = 1.0 gm. of yeast). Even 4 cc. daily did not give a growth rate that one expects. For example, rats receiving 1.0 gm. of yeast daily as the only source of vitamin B averaged 235 gm. in weight for the males and 165 gm. for the females, at the end of the 8th week. Evidently something was lost in the procedure or something was left in the residues.

In a previous paper (2) it was shown that autoclaved yeast is a rich source of antipellagra vitamin, or vitamin G. Since this is the case, then when the A fraction, or vitamin F, is fed in conjunction with autoclaved yeast there should be good growth.

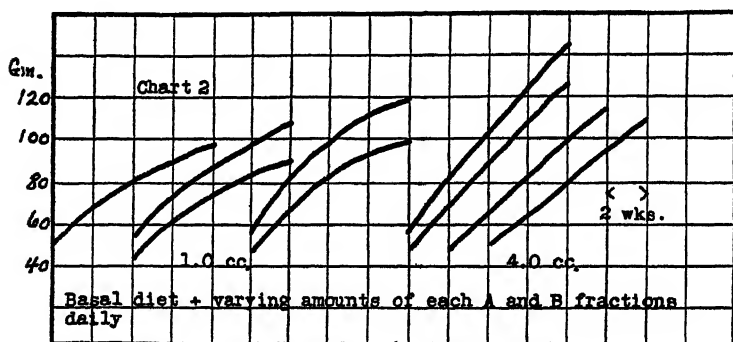


CHART 2.

Chart 3 shows this supplementary value of vitamin F as well as the fact that vitamin G, as would be expected, does not supplement the stimulatory value of autoclaved yeast.

It is now well known that wheat is rich in the antineuritic vitamin, vitamin F, and poor in the growth or antipellagra vitamin, vitamin G. Such being the case one would expect that by adding the B fraction, vitamin G, as in the case of autoclaved yeast, to a diet containing 25 per cent of wheat there would be good growth. The growth curves in Chart 3 confirm this but the growth is not as good as was shown in a previous paper (2) with a similar amount of wheat and autoclaved yeast. Just why this is the case remains to be investigated. However, none of the symptoms of pellagra, such as lesions on body or eye disease, were present, and one

female gave birth to young. This again shows that wheat is relatively poor in the antipellagra vitamin, vitamin G. Another lot of rats was fed a diet containing 25 per cent of wheat as the only source of vitamin B and allowed to remain on this diet until symptoms indicative of pellagra were noted, when the antineuritic

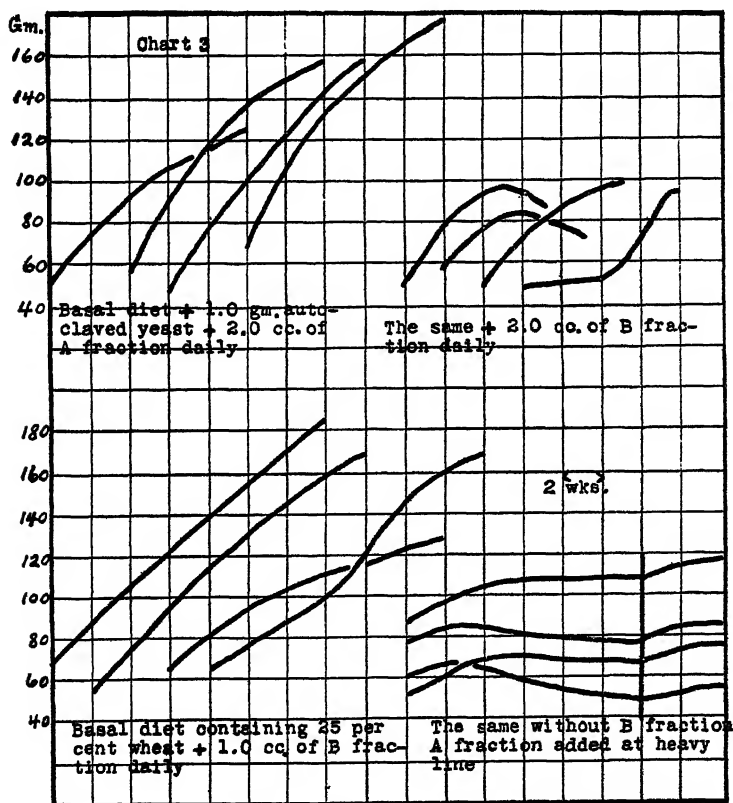


CHART 3.

vitamin, vitamin F, was administered separately. There was very little if any response in growth (Chart 3), neither did the lesions heal, nor the other symptoms disappear. This is as one expects from what we now know regarding the different fractions, and again confirms the fact that vitamin B is composed of at least

two other vitamins, which have been isolated and are distinctly different. There is also a third factor, which is a vitamin, or catalyst, that activates or supplements the other two factors. It seems to be relatively abundant in the yeast residue, and as will be shown later, it is carried over into the fuller's earth residue, either by being in solution or in suspension. In looking about for an explanation as to why good growth was not obtained by

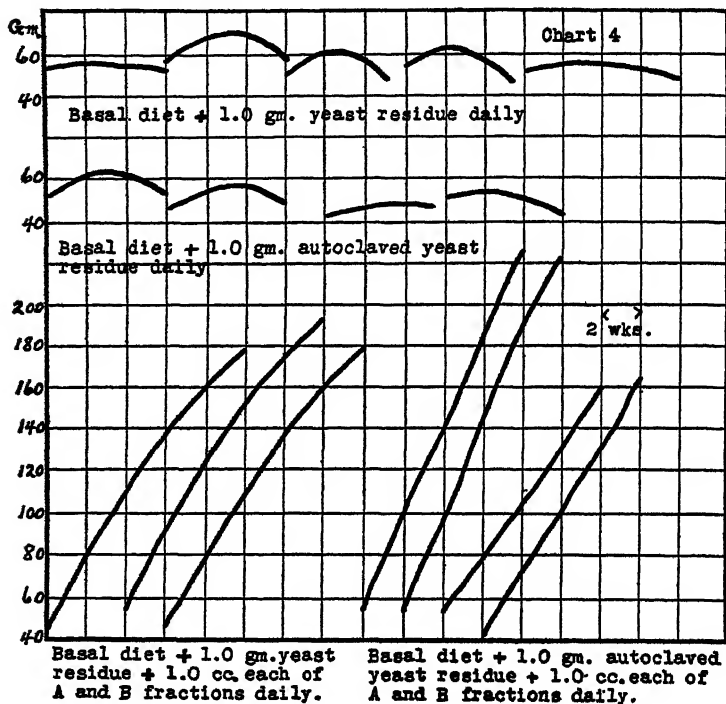


CHART 4.

feeding the two fractions, it was thought that possibly the yeast residue contained some substance that would account for the difference. Since the yeast residue had been thoroughly washed it was inconceivable that a water-soluble substance still remained with the residue. In the meantime the residue had been dried before an electric fan and ground to a powder. This residue was fed to a check lot of rats, but instead of growth (Chart 4) there was a

gradual decline and death in 5 to 6 weeks. However, when the two vitamins of the complex were added to the yeast residue in equal proportions (1 cc. each), and the same fed to rats, there was excellent growth as shown in Chart 4. Similar results were obtained with the autoclaved yeast residue. The investigation was rewarded with the result that here was a substance insoluble in water or 0.01 per cent acetic acid, thermostable, and one which activates, or supplements the two other vitamins of the vitamin B complex. A brief report (5) concerning this substance has been made. Just what this catalyst or vitamin is remains to

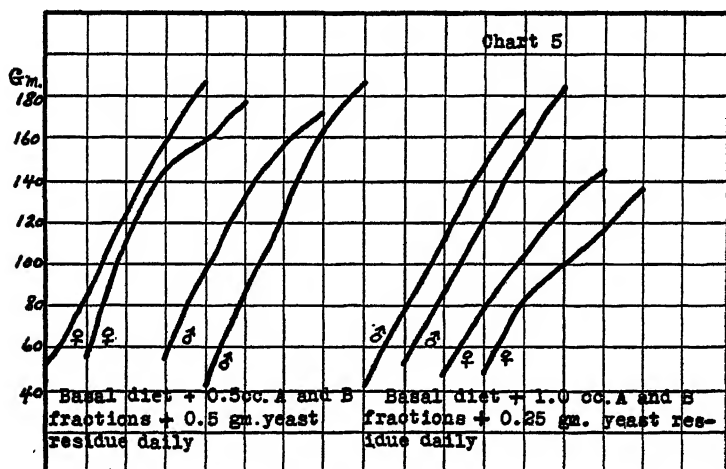


CHART 5.

be determined and is now being investigated. Since this investigation was started Kennedy and Palmer (6) have observed similar results with the yeast residue and their Ration 5 containing the extract equivalent of 15 parts of wheat embryo as the source of vitamin B. The yeast residue is rich in the new factor, as is evidenced by the growth curves in Chart 5. Good growth was obtained on 500 mg. of the autoclaved yeast residue and an equivalent amount of the A and B fractions. This growth is comparable to that obtained with 500 mg. of the original yeast. When 1.0 cc. each of the A and B fractions of the complex was fed in addition to 250 mg. of the autoclaved yeast residue, the growth was good and compared very favorably with the growth curves where 500 mg. of

the autoclaved yeast residue and the equivalent of the A and B fractions were used.

Another series of experiments was run to determine whether the fuller's earth residue contained any of either the fractions A and B, or the new factor. The fuller's earth was fed in the same equivalent amount as the yeast residue. It will be seen from the

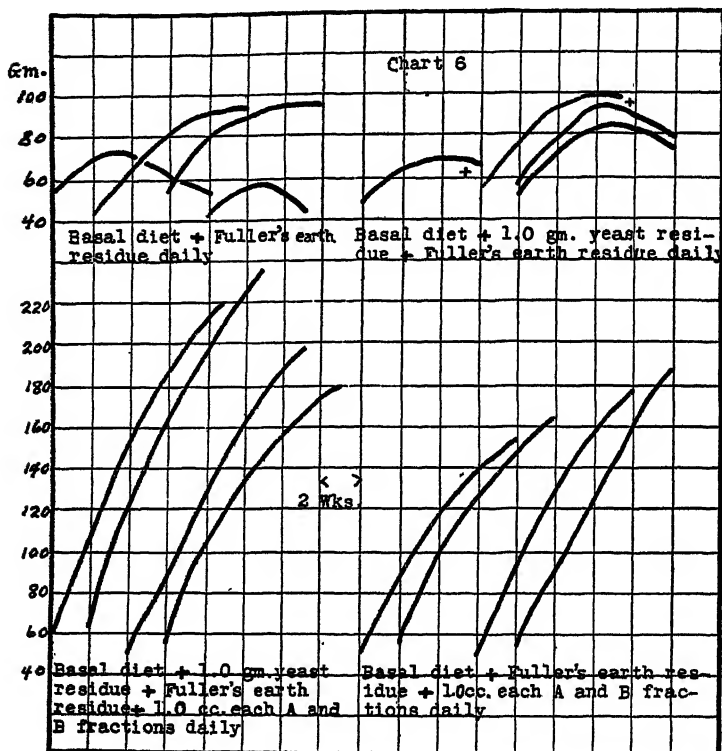


CHART 6.

growth curves (Chart 6) that the earth residue does contain some of one or more of the fractions regardless of the fact that it was extracted twice with barium hydroxide and then washed with distilled water. In another experiment (not reported here), where fuller's earth was used as the only supplement to the basal diet, there was very little if any growth. The only conclusion that can be drawn is that evidently it is always difficult to be sure that

the same procedure gives the same results, as far as the earth residues are concerned, or that one encounters a different grade of fuller's earth. When the fuller's earth residue was fed in conjunction with the yeast residue there was no supplementary effect, but when fed in addition to the A and B fractions, with or without the yeast residue there was good growth. This would indicate that this new factor is soluble in water, or that some of it remains in suspension in the water extract and is then adsorbed on the fuller's earth when so treated and is not removed by the procedure used. The water extract always contained some of the residue in suspension. In another paper this phase of the subject and others will be taken up. The yeast residue seems to have an additional supplementary value to that of the A and B fractions and fuller's earth residue.

SUMMARY.

1. A method of separating the component parts of the vitamin B complex is given.

2. Each of the component parts (vitamins F and G) when fed separately failed to maintain the weight of rats, but when fed in combination there was continuous growth; however, the growth did not approach that produced by an equivalent amount of the original yeast (1 cc. = 1 gm. of yeast).

3. The A fraction, the antineuritic vitamin, supplemented autoclaved yeast, while the B fraction, the antipellagra vitamin, supplemented whole wheat in producing growth in rats. This confirms the previous conclusions that autoclaved yeast is rich in the antipellagra vitamin, vitamin G, and that wheat is rich in the antineuritic vitamin, vitamin F.

4. The deficiency of the antineuritic and antipellagra vitamins, vitamins F and G, as prepared by the above method was found to be a catalyst or a new vitamin resident in the yeast residue, and possibly in the fuller's earth residue.

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THE RELATIVE ANTINEURITIC AND ANTIPELLAGRIC POTENCY OF COW'S MILK.*

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(Received for publication, August 3, 1928.)

Recent investigations have shown that the substance formerly designated as vitamin B in reality is composed of two factors. Some investigators (1, 2) have presented evidence that a third factor exists. In their study of the composite nature of vitamin B, Chick and Roscoe (3) made the observation that milk was a poorer source of the antineuritic factor than of the antipellagic factor. Sherman and Axtmayer (4) in a similar study found that dried skim milk was apparently richer in vitamin G than in vitamin F. In view of the observations of these workers and the great importance of milk as a food, it was thought pertinent to study the relative potency of whole milk in the two known factors of the vitamin B complex.

A method of utilizing the selective adsorption power of fuller's earth was used to separate the different fractions from the acidulated water extract of yeast. The fraction which was adsorbed on the fuller's earth and afterwards removed with barium hydroxide was designated fraction A and, as will be shown later, contained the antineuritic vitamin (vitamin F). That part of the yeast extract not adsorbed by the fuller's earth was designated fraction B. It possessed antipellagic properties and when fed in conjunction with fraction A produced slight growth. In order to make sure that fraction B did not contain any of the antineuritic vitamin it was autoclaved $2\frac{1}{2}$ hours at 15 pounds steam pressure before being used. With polyneuritis- and pellagra-producing basal diets, with additions of fractions A and B, together with

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different levels of milk, it was possible to measure the relative antineuritic and antipellagric vitamin content of milk.

EXPERIMENTAL.

Our general laboratory technique has already been described in detail in a previous paper (5). In all of the experiments the rats were kept individually in wire cages with raised floors. The polyneuritis-producing diet consisted of casein 18, starch 64, McCollum's Salt Mixture 185 4, Crisco 10, agar 2, and cod liver oil 2 parts. The pellagra-producing diet was made up of casein

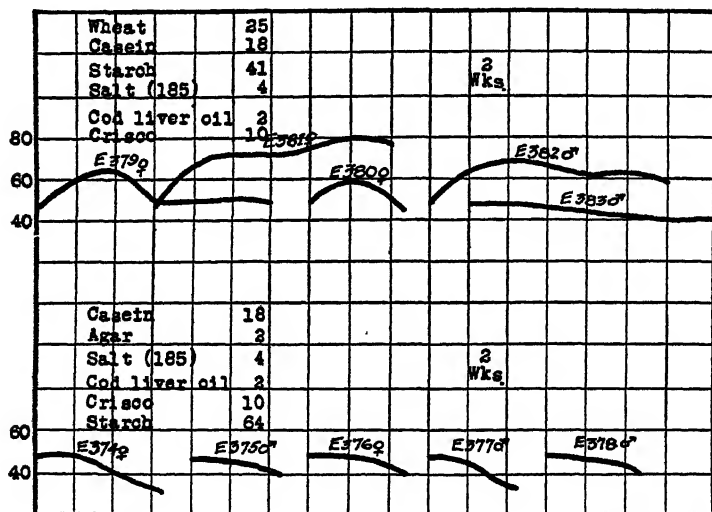


CHART 1.

18, whole wheat 25, starch 41, Salt Mixture 185 4, Crisco 10, and cod liver oil 2 parts. Previous experiments showed that these diets were true to name. The milk used was obtained from station cows receiving a good dairy ration and kept indoors.

The growth curves produced by the separate fractions, when fed in addition to the basal vitamin B-free diet, were reproduced in a previous paper and will not be repeated here. However, to obtain further evidence that these separate fractions were quite pure they were fed to chickens. The basal diet of the chickens

consisted of polished rice 78, casein 16, Salt Mixture 185 4, and cod liver oil 2 parts. Those receiving the A fraction (vitamin F) in addition to the basal diet, maintained their weight and appeared normal, while those that received the B fraction (vitamin G) lost weight and developed polyneuritic symptoms as soon as

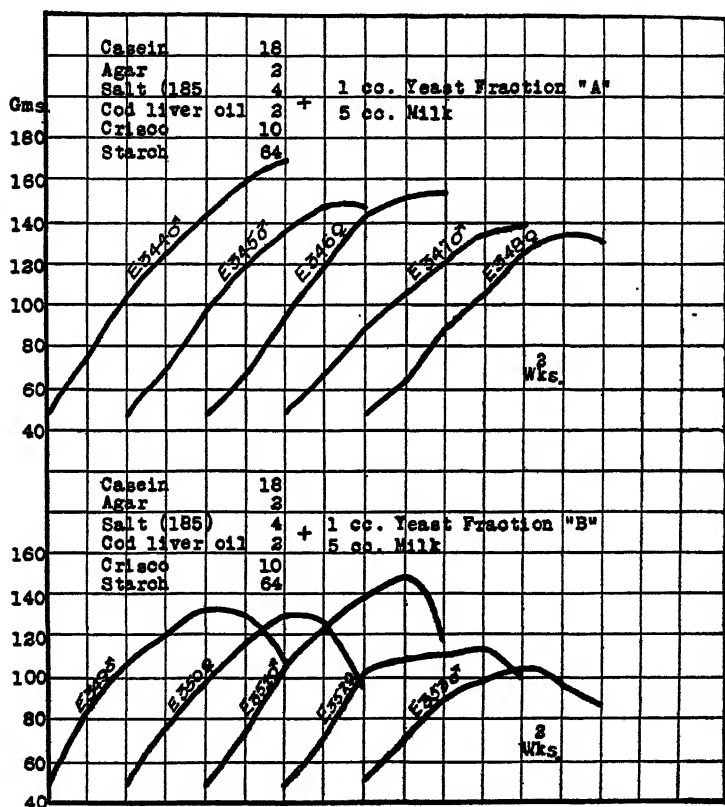


CHART 2.

those of the control group. Those receiving both fractions grew slowly and appeared normal.

Chart 1 shows the growth curves of the control rats on each of the basal diets used. Although the pellagra-producing diet contained 25 per cent of wheat which supplied sufficient of the antineuritic vitamin for normal growth provided the other

fraction of the complex vitamin B was present, there was very little if any growth. In another paper (5) it was shown that autoclaved yeast can supply the other factor.

The results shown in Chart 2 offer evidence that 5.0 cc. of milk daily are a very good supplement to the A fraction (vitamin F)

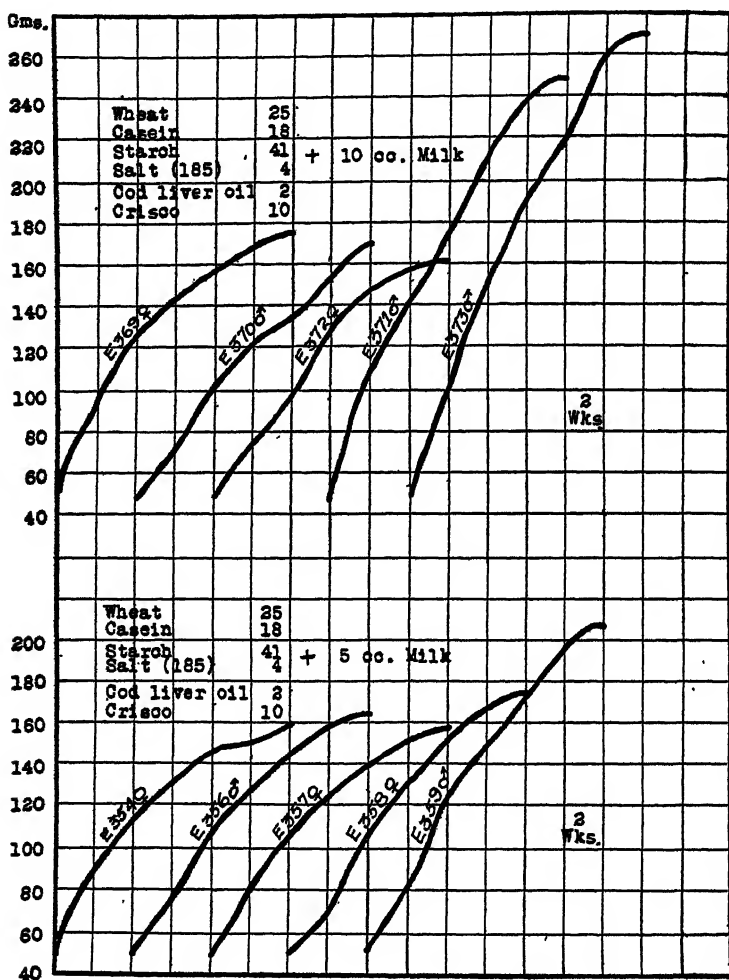


CHART 3.

of the complex. There was good uninterrupted growth for 10 weeks, after which there was a tendency for the curves to flatten. Previous experiments showed that 5.0 cc. of milk did not supply sufficient vitamin F for growth, but in this case where the antineuritic vitamin, vitamin F, was fed in addition to the milk there was growth. This shows that the A fraction supplies the factor that is lacking in milk, *viz.* the antineuritic vitamin. The conclusion to be drawn from this is that milk is richer in the growth or antipellagic vitamin (vitamin G) than in the antineuritic vitamin (vitamin F).

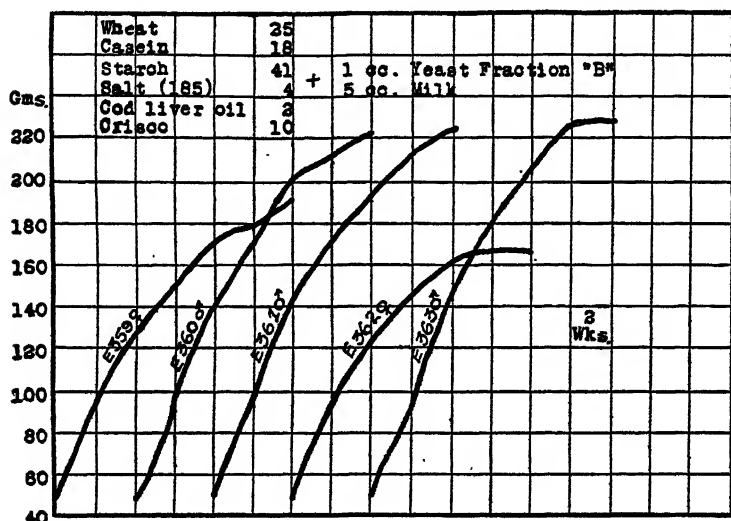


CHART 4.

When 1.0 cc. of the B fraction replaced the same amount of the A fraction and was fed in connection with the vitamin B-free diet and 5.0 cc. of milk there was good growth, as shown in Chart 2, for from 6 to 8 weeks, after which there was a rapid decline. Since the milk was relied upon to supply the antineuritic vitamin and since there was a decline in weight, it follows that milk is deficient in this factor. Evidently the animals had a reserve storage of the antineuritic vitamin and this, together with the small amount that was in the milk, made it possible for them to grow at a good rate until the body supply was exhausted.

Further evidence to the effect that milk contains less of the antineuritic vitamin than of the antipellagric vitamin is given in Chart 3. This basal diet, as has been shown, resulted in practically no growth and produced pellagra after a considerable length of time. One of us in a previous paper (5) presented evidence that autoclaved yeast prevented the pellagra-like symptoms when fed as a supplement to this diet and now when 5.0 cc. of milk were fed as a supplement to the same diet good growth was obtained over the entire experimental period and the rats remained in good condition. It follows then that milk is potent in the antipellagric vitamin (vitamin G) and supplements a cereal diet excellently. The addition of 10 cc. of milk to the diet gave some additional growth over that obtained when 5.0 cc. were fed. This is shown in Chart 3. We believe that 25 per cent of wheat supplies liberally the antineuritic vitamin and that 5.0 cc. of milk carry the minimum of the antipellagric vitamin for growth, and when the two are fed in combination the limiting factor for growth is the vitamin or the vitamins found in the 5.0 cc. of milk. When 5.0 cc. of milk were supplemented with 1.0 cc. of the B fraction excellent growth was obtained which confirms our belief (Chart 4). This is clearly shown when Chart 3 is compared with Chart 4.

No attempt was made to evaluate the potency of milk in the third factor of the vitamin B complex.

CONCLUSIONS.

Milk from cows under winter feeding conditions is potent in the antipellagric vitamin (vitamin G) and relatively poor in the antineuritic vitamin (vitamin F).

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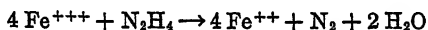
A GASOMETRIC METHOD FOR DETERMINATION OF REDUCING SUGARS, AND ITS APPLICATION TO ANALYSIS OF BLOOD AND URINE.

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(Received for publication, August 14, 1928.)

The methods here described depend on the quantitative reduction of potassium ferricyanide by sugar in an alkaline solution, and the gasometric determination in the Van Slyke-Neill apparatus (1924), of the nitrogen evolved when the excess ferric salt is permitted to react with hydrazine.



The method is exact, rapid, requires only simple and stable reagents, and no standards. Oxalate and fluoride used as anticoagulants in blood do not interfere. The blood sugar determinations are made after removal of proteins by the Folin-Wu tungstic acid precipitant.

One determines in a control analysis without sugar the pressure p_0 , exerted by the N_2 evolved when all the ferricyanide in the reagent reacts with hydrazine in the gas apparatus. The diminished pressure, p_1 , is then determined, which is exerted by the N_2 evolved from reaction of hydrazine with the amount of ferricyanide left unchanged, after part has been reduced to ferrocyanide by the sugar. The pressure difference, $p_0 - p_1$, is proportional to the amount of sugar present. Contrary to most analyses in the Van Slyke-Neill gas apparatus, the zero reading in this case is near the top of the manometer; the more sugar there is in the sample the lower is the manometer reading obtained in its analysis, because the sugar destroys the ferricyanide, of which the N_2 pressure is a measure.

Ferricyanide as a reagent for determining reducing sugars has

an advantage over copper reagents, in that the ferrocyanide produced by reaction with sugar is not readily reoxidized by the air. The ferricyanide solution partially reduced by heating with sugar may be permitted to stand for several hours at room temperature in contact with air without influencing the results obtained when the analysis is completed by determination of the excess ferric iron.

Hagedorn and Jensen (1918) first made use of the reduction of potassium ferricyanide for the determination of blood sugar. Ray and Sen (1912) have shown that potassium ferricyanide reacts quantitatively with hydrazine in an alkaline solution, liberating free nitrogen gas according to the above equation. The writers published a preliminary outline of the present method 2 years ago (Van Slyke and Hawkins, 1926).

MACRO DETERMINATION OF REDUCING SUGAR IN BLOOD.

This determination is performed with a volume of Folin-Wu blood filtrate equivalent to 0.3 cc. of blood. We have called it the macro method to differentiate it from the micro method, described later, which requires still less material.

Reagents for Macro Blood Sugar Determination.

Potassium Ferricyanide Reagent.—8 gm. of potassium ferricyanide; 75 gm. of anhydrous potassium carbonate, and 75 gm. of potassium bicarbonate are dissolved in water and made up to 1 liter.

The potassium carbonate and bicarbonate are dissolved in approximately 750 cc. of warm distilled water. The potassium ferricyanide is dissolved in about 100 cc. of distilled water and added to the carbonate-bicarbonate solution. After the mixed solution has cooled to room temperature, it is made up to a liter with distilled water and filtered. Filtration is necessary even though the solution appears perfectly clear. The solution is kept in a dark-colored, glass-stoppered bottle. It will keep indefinitely in the dark. The reagents do not need to be weighed with any great degree of accuracy, as the control that is run when sugar determinations are made determines the amount of ferricyanide present.

Alkaline Hydrazine Solution.—A saturated solution of hydrazine sulfate is made by dissolving 25 gm. of the substance in

500 cc. of warmed water, and letting the excess crystallize out on cooling.

A 40 per cent solution of the purest grade NaOH is prepared by dissolving 200 gm. in water and diluting to 500 cc. We have used Merck's "Reagent sodium hydroxide from sodium." Some other commercial preparations have been found to contain impurities which affect the results.

The alkaline hydrazine solution is made by mixing equal volumes of the saturated hydrazine sulfate and of the 40 per cent sodium hydroxide solution. The mixed solution kept at room temperature has shown no deterioration within 3 months. How much longer it will keep we have not ascertained.

Tungstic Acid Solution (Mixed Reagents of Folin and Wu (1919)).—1 volume of 10 per cent sodium tungstate ($\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$) is mixed with 8 volumes of N/12 sulfuric acid. The acid solution is not permanent, but can be used for 2 weeks. A slight precipitate appears in a shorter time, but without diminishing significantly the effectiveness of the solution as a precipitant for the blood proteins.

Procedure for Macro Blood Sugar Determination.

Precipitation of Blood Proteins.—A flask calibrated to contain tenfold the volume of the blood sample is filled about two-thirds full with the tungstic acid solution, into which the blood sample is pipetted. Sufficient additional tungstic acid solution is added to make the volume up to the mark. An alternative procedure is to add the blood sample to 9 volumes of tungstic acid solution. The mixture is thoroughly shaken, and after 2 minutes or longer is filtered through a dry filter.

Folin and Wu (1919) originally added separately to each blood sample the water, 10 per cent sodium tungstate solution, and sulfuric acid ($\frac{2}{3}$ N), of the above tungstic acid solution. The use of a single solution in which the reagents are already combined simplifies the procedure, and also has been found to yield a somewhat larger volume of filtrate. The filtrates obtained can be used for any of the determinations applicable to the Folin-Wu blood filtrate. The acidified tungstate solution has a slight disadvantage in that it does not last indefinitely; but the trouble involved in preparing it fresh once in 2 weeks is negligible.

Reduction of Ferricyanide by Blood Filtrate.—3 cc. of filtrate are pipetted into a Pyrex test-tube (14 by 125 mm. outside measure) followed by 1.5 cc. of ferricyanide solution.¹ Both solutions must be measured accurately from Ostwald bulb pipettes with capillary stems. The ferricyanide solution should be added last, in order to facilitate its mixture with the lighter filtrate. The tube is closed by a 1-hole rubber stopper (Fig. 1), through which passes



FIG. 1. Test-tube used for reduction of ferricyanide by sugar.

a capillary tube 2 cm. long of 0.6 mm. bore. The tube is shaken gently to mix the two solutions, and is then immersed for 20

¹ It is necessary to use a special 1.5 cc. pipette, with stem of 1 mm. bore, for precise measurement of the ferricyanide. Such pipettes can be obtained from the makers of the manometric apparatus. In case 1.5 cc. pipettes are not at hand, 1 cc. of a ferricyanide-carbonate solution containing, per liter, 12 gm. of $K_3Fe(CN)_6$, 100 gm. of K_2CO_3 , and 100 gm. of $KHCO_3$ may be added from a 1 cc. Ostwald pipette with a capillary stem. It is advisable to use the same pipette for the control analysis, in which p_0 is determined, and for the unknown. If this is done the final result is unaffected by any error in the calibration of the pipette. When only 1 cc. of the above 1.2 per cent ferricyanide solution is added, instead of the usual 1.5 cc. of 0.8 per cent ferricyanide, the factors in Table II for the macro blood method require multiplication by $\frac{4.0}{4.5} = 0.909$, in order to apply, since the 3 cc. of filtrate are diluted only to 4.0 instead of 4.5 cc. with ferricyanide reagent; *e.g.*, the factor for 20° is thus changed from 1.457 to 1.325.

minutes in a boiling water bath. Usually a series of tubes is heated at once; it is convenient to use a cylindrical copper rack,² such as is employed by bacteriologists, to hold the tubes. The solutions are cooled by immersing the tubes in cold running water for 3 minutes and then in a beaker of water at room temperature.

After they reach room temperature the solutions are resaturated with air by shaking them vigorously for 1 minute. Six or eight tubes are clasped with the fingers so that the tops of the tubes are in the palm of the hand. This insures against the stoppers coming out and also prevents warming the solutions by the heat of the hand. The capillary openings through the stoppers must not be closed. They permit the air, driven out during the heating, to reenter when the tubes are cooled and shaken, while the narrowness of the bore prevents more than an occasional drop of solution from passing out and being lost. The loss of a drop or two does not matter, since an aliquot of the solution is used for the final analysis. After the solutions are thus aerated it is our custom to let them stand until analyzed in a beaker of water at room temperature, to prevent sudden temperature changes.

It is essential that the resaturation with air shall be complete: the amount of dissolved air in the solution exerts about 60 mm. of pressure in the subsequent manometer reading, and it is essential to have it the same in the control tube and all the filtrate tubes. In practically every case in which analysts to whom we have given this blood sugar method have had trouble with it, the cause has been failure to make the resaturation with air complete. In consequence the solutions in the tubes continue to absorb air slowly as they stand waiting for the gasometric analysis, and those tubes with blood filtrate which stand longer than the control, register less fall in N_2 pressure (measured as $p_0 - p_1$) than they should, and lower sugar values.

The ferricyanide present in the reagent is enough to oxidize 350 mg. of glucose per 100 cc. of blood. If more is present, the filtrate plus ferricyanide solution will, after heating, be entirely colorless, all the yellow ferricyanide having been reduced to colorless ferro-

² These copper racks may be obtained from Eimer and Amend, New York, catalogue No. 32002, or from Arthur H. Thomas Company, Philadelphia, catalogue No. 9488.

cyanide. In such a case another portion of blood filtrate is diluted with an equal volume of water, and the analysis is repeated with the diluted filtrate. In calculating the results the factor in Table II must then be doubled.

Gasometric Determination of Excess Ferricyanide.—Sufficient alkaline hydrazine solution is run from the cup of the Van Slyke-Neill apparatus down into the mercury-filled chamber to reach exactly to the 2 cc. mark on the chamber. The stop-cock of the chamber is closed and the excess hydrazine solution is sucked out of the cup, in which 1 to 2 cc. of mercury are then placed. Through this mercury seal 3 cc. of the previously heated and re-aerated sugar-ferricyanide solution are then measured into the chamber of the apparatus from a rubber-tipped stop-cock pipette as described in a former paper (Van Slyke, 1927; see Fig. 3 of that paper). The stop-cock is sealed by admitting just enough mercury to fill its bore; mercury drops are not permitted to stream down through the solution in the chamber, as they might reduce an appreciable amount of the ferricyanide solution before it is mixed with the hydrazine. The mercury in the chamber is lowered to the 50 cc. mark. When the ferricyanide and hydrazine solutions mix, their reaction is practically instantaneous, as evidenced by the ebullition of nitrogen gas. The evacuated chamber is shaken rapidly 1 minute to complete the evolution of the gas, and the volume of the latter is then reduced to 0.5 cc. (If the chamber is shaken slowly $1\frac{1}{2}$ minutes may be needed.) The pressure in the manometer is read, p_1 if a blood filtrate is analyzed, p_0 if the determination is that of the control without sugar. The solution is then ejected from the chamber, and the apparatus is ready for the next determination.

Preliminary Determination of p_0 .—The control determination is performed with 0.9 per cent NaCl solution in place of the blood filtrate, because this solution has the same solvent power for air as the Folin-Wu filtrate. The dissolved air provides part of the pressure observed in the analysis, and hence must be equal in determination of p_0 and p_1 . In all details, heating, aeration, etc., the control determination is carried through exactly like the analysis of the blood filtrate, described below. One control provides the p_0 for an entire series of blood analyses.

Calculation of Results of Macro Blood Analysis.

The $p_0 - p_1$ reading multiplied by the proper factor from Table II gives directly the blood sugar in mg. per 100 cc.

If the temperature, observed on the thermometer in the water jacket of the extraction chamber, has changed at the time of the p_1 reading from the temperature at the p_0 determination, the p_0 value used in the calculation is corrected as indicated by Table I.

Remarks on Gasometric Technique.—It is not necessary to wash the chamber of the gas apparatus between the successive determinations of a series. A great excess of alkaline hydrazine solu-

TABLE I.
Correction to p_0 for Temperature Change.*

Temperature range.	Increase of vapor tension of water per 1° temperature rise.	Macro blood analyses.		Micro blood analyses.		Urine analyses.	
		Increase of N_2 pressure of control analysis per 1° temperature rise.	Total p_0 correction per 1° temperature change.	Increase of N_2 pressure of control per 1° temperature rise.	Total p_0 correction per 1° temperature change.	Increase of N_2 pressure of control per 1° temperature rise.	Total p_0 correction per 1° temperature rise.
°C.	mm.	mm.	mm.	mm.	mm.	mm.	mm.
15-20	0.7	1.0	1.7	0.5	1.2	1.1	1.8
20-25	1.2	1.0	2.2	0.5	1.7	1.1	2.3
25-30	1.6	1.0	2.6	0.5	2.1	1.1	2.7

* Add correction to p_0 if temperature, observed at p_1 reading, is higher than that at the p_0 reading; subtract correction if temperature at p_1 reading is lower than at p_0 reading. The "total p_0 correction" in each case is the sum of the rise in vapor tension given in the second column plus the increase in N_2 pressure of the control per 1° temperature rise.

tion is used for each determination, and effectively cleans the chamber for the next. The gasometric determinations in a series can be run off at the rate of one every 3 minutes. A technician in this laboratory has in routine work finished 35 complete blood sugar analyses in 2½ hours.

It is necessary that the hydrazine and ferricyanide-sugar solutions be run into the chamber of the gas apparatus in the order directed, the hydrazine first, then the ferricyanide-sugar solution. When thus added, the two solutions divide into layers, with the heavier alkaline hydrazine solution remaining on the bottom in

TABLE II.

Factors by Which N_2 Pressure Fall, $p_0 - p_1$, in Millimeters Is Multiplied to Calculate Reducing Sugar of Blood or Urine in Terms of Glucose.

Temperature of gas chamber.	Blood analyses. Gas pressures measured at 0.5 cc. volume.		Urine analyses. Gas pressures measured at 2.0 cc. volume.	
	Factors to calculate mg. sugar per 100 cc. blood.		Factors to calculate per cent sugar in urine.	
	Macro blood method.	Micro blood method.	Urine diluted 1:20.	Urine diluted 1:50.
°C.				
10	1.508	4.37	0.0136	0.0340
11	03	5	5	0.0338
12	1.498	4	5	7
13	93	2	4	6
14	87	1	4	5
15	82	4.29	4	4
16	77	8	3	2
17	72	7	3	1
18	67	5	2	0
19	62	3	2	0.0329
20	57	2	2	8
21	52	1	1	7
22	47	4.19	1	6
23	42	8	0	5
24	37	6	0	4
25	32	5	0.0129	2
26	28	4	9	1
27	23	2	9	0
28	18	1	8	0.0319
29	14	4.09	8	8
30	09	8	7	7
31	04	7	6	6
32	00	5	6	5
33	1.395	4	6	4
34	91	3	5	3

The factors are calculated as follows:

Let P = mm. pressure fall per 1 mg. of glucose at 25° , under the conditions of the analysis.

V_b or V_u = cc. volume of blood or urine sample represented in the portion transferred to the gas apparatus.

t = temperature in $^\circ\text{C}$. in water jacket of gas chamber.

contact with the mercury. The ferricyanide is completely reduced by the hydrazine before coming into contact with the mercury. If the ferricyanide were added first, contact with the mercury in the chamber would partially reduce it before it mixed with the hydrazine.

Before a series of determinations is started it is advisable to run 2 or 3 cc. of the alkaline hydrazine solution into the chamber of the gas apparatus, and evacuate and shake the latter for 2 or 3 minutes, in order to make sure that there are no impurities present which can oxidize hydrazine.

This treatment has sufficed to prepare the apparatus after any previous use except micro-Kjeldahl analyses by the hypobromite method (Van Slyke, 1926-27). The mercuric bromide which apparently clings to the walls of the chamber, or to the rubber connecting tube below it, reacts with hydrazine for a long time. This bromide is readily dissolved by a strong solution (saturated to half saturated) of sodium bromide. 10 or 15 cc. of the latter are run into the chamber of the gas apparatus, and the mercury is lowered until the solution appears in the glass tube below the flexible rubber joint underneath the chamber. If the apparatus has been used for many analyses with hypobromite, it may be necessary to let the sodium bromide solution stand thus in it for 2 hours. The chamber is then washed with distilled water and with alkaline hydrazine solution.

F = factor by which observed $p_0 - p_t$ value is multiplied to obtain mg. of sugar per 100 cc. of blood, or gm. per 100 cc. of urine.

$$\text{Then, for blood: } F = \frac{1}{P} \times \frac{100}{V_b} \times \frac{273 + 25}{273 + t}$$

$$\text{For urine: } F = \frac{1}{P} \times \frac{100}{1000 V_u} \times \frac{273 + 25}{273 + t}$$

The values of V_b are, for the macro blood analysis 0.200 cc., for the micro blood analysis 0.077 cc. The corresponding values of P are 349 and 313 mm. (see Fig. 4 and Table VI), measured at 0.5 cc. of gas volume.

The value of V_u for analysis of urine 20-fold diluted is 0.075 cc.; for 50-fold diluted urine V_u is 0.03 cc. The corresponding value of P is 103.0 mm. (see Fig. 4 and Table VII), measured at 2.0 cc. gas volume.

In case a blood filtrate, because of great sugar content, is diluted twice as much as prescribed in the directions for usual analyses, so that V_b is halved, the value of the factor F given in Table II is doubled.

Scale for Direct Reading of Sugar Values.

Calculation can be obviated by preparing on a strip of millimeter paper a scale on which, when it is fastened beside the manometer tube, sugar percentages can be read off directly from the level of the mercury column at the time of the p_1 readings with sufficient accuracy for most purposes. Such a scale is exact only for one temperature. However, if two scales are prepared, one for 20° and one for 25°, they can be used to cover the temperature range from 17.5–27.5°, with a maximum calculation error of less than 1 per cent.

If, for example, a scale is prepared for the macro blood sugar method at 20°, an interval indicating 10 mg. per cent of blood sugar corresponds to $\frac{10}{1.457} = 6.86$ mm. (see Table II). The scale is

made for sugar values up to 350 mg. per cent by marking off 35 intervals, at 6.9, 13.7, 20.6 . . . *etc.* mm. from the top, and marking the points 10, 20, 30 . . . *etc.*, showing the mg. per cent of blood sugar indicated by each point. Each 10 mg. interval is divided into five subdivisions indicating differences of 2 mg. per cent of sugar. The scale is attached by a piece of adhesive tape or other device to the board beside the manometer tube, with the zero point at the top of the scale placed level with the p_0 reading on the manometer. Then when a sugar-ferricyanide solution is analyzed, the reading on the scale opposite the mercury meniscus indicates at once the mg. per cent of blood sugar.

MICRO METHOD FOR ANALYSIS OF 0.2 CC. SAMPLES OF BLOOD.

Reagents for Micro Blood Analysis.

Ferricyanide Solution.—This contains, like the above ferricyanide, 75 gm. each of K_2CO_3 and $KHCO_3$, but only 4 gm. of $K_3Fe(CN)_6$, per liter.

Alkaline Hydrazine Solution.—Same as above.

Tungstic Acid Solution.—2 volumes of the tungstic acid solution described above are diluted with 3 volumes of distilled water.

Procedure for Micro Blood Analysis.

Measuring Blood Sample and Removing Proteins.—When samples of capillary blood are to be analyzed by the micro method, a

sufficient number of rubber-stoppered centrifuge tubes is prepared, each containing 5 cc. of the dilute tungstic acid precipitating reagent described above. The blood drops forming on the incised finger or ear lobe are drawn by capillary attraction into a 0.200 cc. capillary pipette, which is at once emptied into one of the test-tubes. The pipette is rinsed twice by drawing the tungstic acid up into it. The test-tube is then stoppered and shaken. After any convenient interval the tube is centrifuged for 5 minutes. The 0.200 cc. pipette is made from a capillary tube of about 1 mm. bore, and is calibrated by weighing 2.71 gm. of mercury in the dry pipette.

Analysis of Blood Filtrate.—The tip of a simple Ostwald pipette, calibrated to deliver 3 cc., is covered with absorbent cotton to serve as a filter, a point of technique introduced by Somoogy (1926). The cover is prepared by placing the tip of the pipette on a small, thin square of absorbent cotton held in the fingers of one hand, and twisting the pipette until the cotton is wound tightly about the tip. Through this filter the pipette is filled with supernatant solution from the centrifuge tube. The filter is then removed from the pipette tip, and 3 cc. of filtrate are delivered into a Pyrex glass test-tube (14 × 125 mm.). 1.5 cc. of the ferricyanide reagent are then added.

A control tube is set up containing 1.5 cc. of the ferricyanide reagent and 3.0 cc. of a 0.4 per cent solution of sodium chloride.

The procedure from this point is exactly the same as in the macro method described above.

Gasometric Determination of Sugar in Blood Containing Ether.

The blood from an anesthetized subject may contain enough ether to give a vapor pressure of 40 to 50 mm. when the Folin-Wu filtrate, after reaction with ferricyanide, is shaken in the Van Slyke-Neill apparatus. The effect is to make the sugar results come out too low; i.e., p_1 is too high on account of the ether, hence $p_0 - p_1$, which serves as a measure of the sugar, is made too small.

The error is prevented as follows: A mark is etched to indicate 3 cc. content on the small test-tube used for the reaction of ferricyanide with blood filtrate. After the 3 cc. of filtrate have been placed in the tube, the filtrate is heated with a micro burner, and

is boiled for 2 minutes to drive off the ether. The filtrate is then cooled, and is diluted back to the 3 cc. mark.

Or if more convenient, the tube may be weighed to within 0.01 gm. before the boiling; the water which has been driven off is then replaced by weight.

After the ether has been driven off and the water replaced, ferricyanide reagent is added, and the analysis is continued as usual.

DETERMINATION OF SUGAR IN URINE.

The procedure here outlined is designed for use with urines, such as those encountered in diabetes, in which the significant variations in glycosuria are gross enough to be satisfactorily shown by measurement of the total reducing substances. The method is sensitive to 0.02 per cent of glucose in solution. Normal urine may show reducing power equal to that of a 0.2 per cent glucose solution, sufficient to cause a 15 mm. fall in the manometer reading, but due almost entirely to non-fermentable urinary substances (Eagle, 1926-27; Hawkins, MacKay, and Van Slyke, 1928), which have no apparent relationship to carbohydrate metabolism. In view of this fact, a technique more sensitive than that presented appears to have no significant application when only the total reducing substances are determined.³

Reagents for Urine Sugar.

Ferricyanide Solution.—28 gm. of potassium ferricyanide and 75 gm. each of K_2CO_3 and $KHCO_3$ are dissolved in water, made up to 1 liter, and filtered. The solution is kept in a stoppered bottle of dark glass.

Alkaline Hydrazine Solution.—Same as for blood sugar.

Procedure for Urine Sugar.

Dilution of Urine.—1 cc. of urine is diluted with water ordinarily to 20 cc. In urine so diluted the ferricyanide reagent used will determine up to 3.5 per cent of glucose. In case the sugar content is known to be very high, 1 cc. of urine is diluted to 50 cc.,

³ For use in determinations of fermentable sugar in urine we shall in a later paper present a gasometric procedure sensitive to glucose in 0.001 per cent concentration.

so that glucose up to about 9 per cent can be determined. Dilution is the only preliminary treatment of the urine required. Even albumin does not affect the determination significantly.

Reduction of Ferricyanide by Diluted Urine.—2 cc. of the diluted urine are measured into a Pyrex glass test-tube (14×125 mm.), and 2 cc. of the ferricyanide solution are added. The solutions are mixed, heated 20 minutes, cooled, and aerated, all as described above for blood analyses. A slight, flocculent, permanent precipitate, presumably of calcium and magnesium carbonate, appears when the urine filtrate and ferricyanide reagent are mixed, but it does not interfere in any way with the analysis.

Gasometric Determination of Excess Ferricyanide.—1 drop of caprylic alcohol, to prevent foaming, is run into the chamber of the Van Slyke-Neill apparatus, followed by 2 cc. of alkaline hydrazine solution, measured as described above for blood analyses. 3 cc. of urine-ferricyanide mixture are added through a mercury seal, as described above for the blood analysis, and the chamber of the apparatus is shaken 1 minute to complete the evolution of the nitrogen gas. The gas volume is reduced to 2.0 cc. and the pressure is read on the manometer, p_1 if urine filtrate is analyzed, p_0 if the determination is a control. The air dissolved in the reagents exerts about 13 mm. pressure; the N_2 from all the ferricyanide in the control exerts about 300 mm.

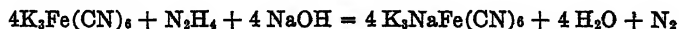
The "Remarks on Gasometric Technique" made above in connection with the blood sugar determination apply also to the urine analysis.

Calculation consists in multiplying the observed $p_0 - p_1$ value by the factor in Table II, p_0 being corrected by Table I if temperature changes intervene between the p_0 and p_1 readings. A scale, as described in connection with the blood analysis, may be used to obviate calculation.

EXPERIMENTAL.

Quantitative Reaction of Ferricyanide with Hydrazine in Gas Apparatus.

The reaction



was tested in the Van Slyke-Neill manometric blood gas apparatus to determine whether sufficiently exact quantitative results could

be obtained to afford a basis for the sugar method. The ferricyanide solution analyzed was made by weight to contain 2.750 gm. of $K_3Fe(CN)_6$ and 5 gm. of Na_2CO_3 per liter. 2 cc. portions were mixed in the chamber of the gas apparatus with 2 cc. of the alkaline hydrazine solution described above for blood and urine sugar analyses. The chamber was evacuated and shaken 1 minute, and the gas pressure was observed on the manometer after the

TABLE III.

Results Showing Yield of N_2 from Reaction of Ferricyanide with Hydrazine, and Stability of Ferricyanide at 100°C. in Alkaline Carbonate Solution.

Ferricyanide present in the 2 cc. of solution used = 5.50 mg.

Theoretical N_2 yield = 0.1170 mg.

Treatment of ferricyanide solution before analysis.	Pressure at 0.5 cc. volume of N_2 gas.	Temperature.	‘ N_2 evolved.	
	mm.	°C.	mg.	per cent of theoretical
None.....	154.2	24.0	0.1166	99.6
“	156.0	24.0	0.1178	100.6
“	153.2	24.0	0.1158	98.9
“	158.2	24.5	0.1192	101.8
Heated in boiling water 15 min. Stood 60 min. at room temperature.....	155.2	24.0	0.1172	100.2
Heated in boiling water 15 min. Stood 90 min. at room temperature.....	155.2	24.0	0.1172	100.2
Heated in boiling water 120 min. Stood 30 min. at room temperature.....	156.6	24.0	0.1182	101.0

gas volume had been brought to 0.5 cc. in the chamber. The zero point on the manometer was obtained by control analysis of a 0.5 per cent Na_2CO_3 solution. The results are given in Table III.

It is apparent from the results in Table III that the reaction of alkaline hydrazine solution with potassium ferricyanide, in concentrations such as are used in our blood analyses, is quantitative, and that heating such as is used in the sugar determinations does not of itself decompose any measurable amount of ferricyanide.

Resistance of Ferrocyanide to Reoxidation by Air.

Portions of a solution like the above, except that it contained potassium ferrocyanide instead of ferricyanide, were heated in the usual test-tubes for an hour. 2 cc. portions were then shaken with alkaline hydrazine solution in the Van Slyke-Neill apparatus. No measurable amounts of nitrogen gas were evolved. This negative result indicates that no ferrocyanide had been changed to ferricyanide.

TABLE IV.

Influence of Carbonate Mixture on Amount of Reducing Substances Determined in Blood Filtrate with 15 and 30 Minutes Heating.

Ferricyanide reagent.			Sugar per 100 cc. blood calculated as glucose.*		Reducing substances per 100 cc. blood by Folin-Wu method.
No.	Na ₂ CO ₃ per l.	NaHCO ₃ per l.	15 min.	30 min.	
	gm.	gm.	mg.	mg.	mg.
1	5	0	100	101	
2	40	0	122	123	
3	20	20	98	97	96
4	30	10	116	113	
5	10	30	111	95	

* Each blood sugar value was estimated from the ratio between fall in N₂ pressure, shown in Fig. 3, caused by heating a given ferricyanide reagent with blood filtrate for a given time, and fall in N₂ pressure shown in Fig. 2, caused by heating the same reagent for the same time with glucose. Hence

$$\text{mg. of glucose per 100 cc. of blood} = 100 \times \frac{(p_0 - p_1) \text{ per 0.2 cc. blood}}{(p_0 - p_1) \text{ per 0.2 gm. glucose}}$$

both $(p_0 - p_1)$ values being determined with the same reagent heated the same time.

Effect of Carbonate Buffer Mixture on Amount of Ferricyanide Reduced by Glucose and by Blood Filtrate.—Hagedorn and Jensen (1923) in their method use sodium carbonate to make the ferricyanide reagent alkaline for the reduction by sugar. Somogyi (1926) in his modification of the Shaffer-Hartmann (1920-21) method has shown that the alkalinity of the copper solution affects markedly the amount of copper that is reduced by the sugar. We have used varying amounts of carbonate and bicarbonate in the ferricyanide reagents to determine the conditions under which ferricyanide would undergo relatively the most reduction by the

glucose and the least by the non-glucose substances in blood filtrate.

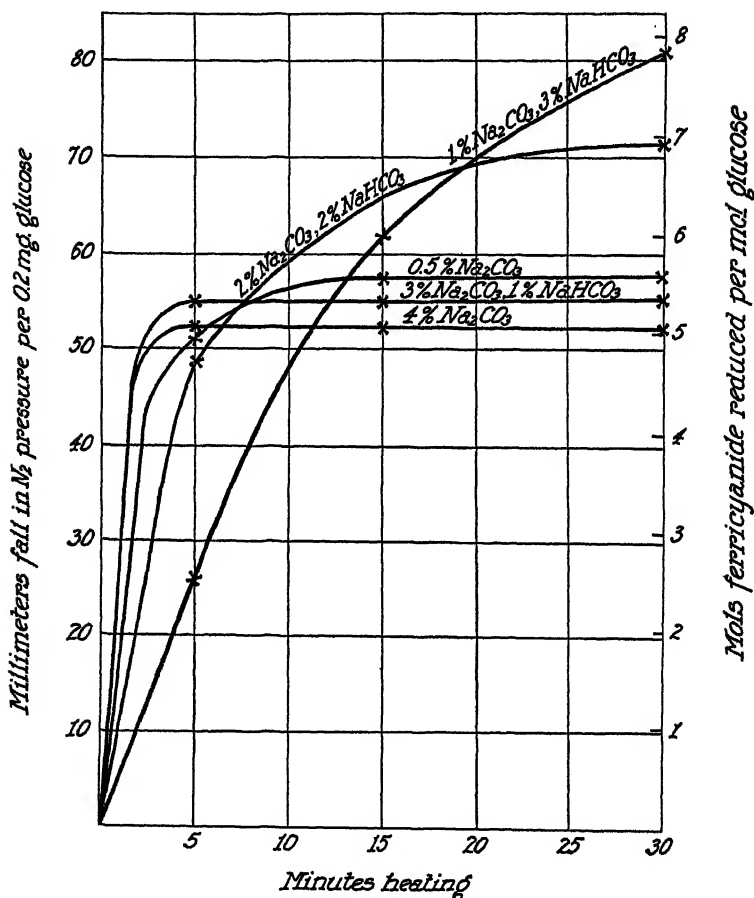


FIG. 2. Time curve of reduction by glucose of ferricyanide reagents, containing 0.8 per cent of potassium ferricyanide and varying amounts of sodium carbonate and bicarbonate. The N_2 pressure changes are measured at 25° and 0.5 cc. volume.

Various reagent solutions were made up with 8 gm. of potassium ferricyanide per liter in each and varying amounts of sodium carbonate and bicarbonate, as shown in Table IV.

Reduction by Glucose—1.5 cc. of each ferricyanide reagent solution were mixed with 3 cc. of 0.03 per cent glucose solution and the mixture in the usual test-tubes was heated in a boiling water bath. Determinations were

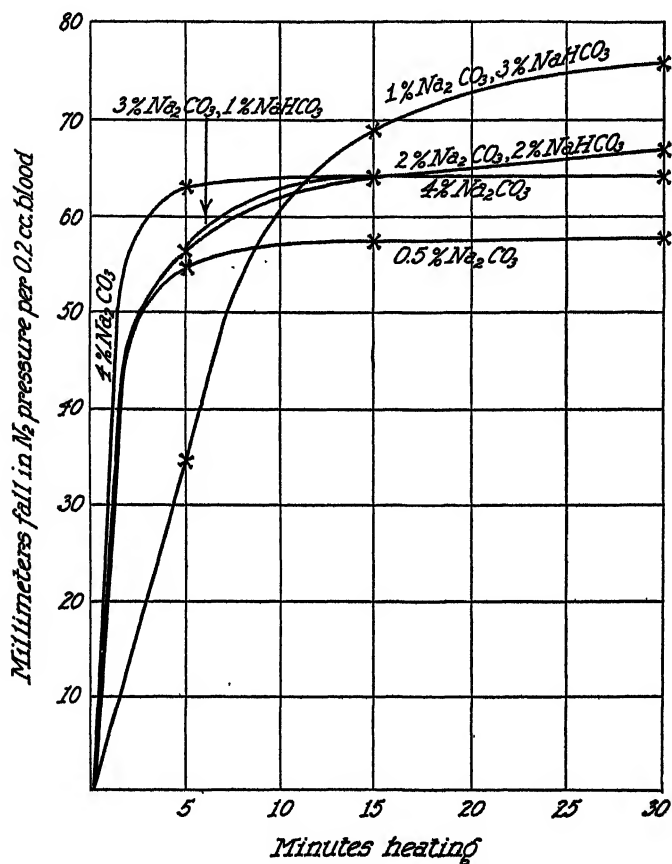


FIG. 8. Time curves of reduction, by blood filtrate, of ferricyanide reagents containing 0.8 per cent of potassium ferricyanide and varying amounts of sodium carbonate and bicarbonate. The N_2 pressure changes are measured at 25° temperature and 0.5 cc. volume.

made, as described for blood sugar determinations, after 5, 15, and 30 minutes heating. Controls for the corresponding ferricyanide-sugar solutions, made by mixing 1 volume of ferricyanide reagent with 2 volumes of distilled water, were run at the same time to determine p_0 readings.

The results are given in Fig. 2. The samples used for gas analysis represented each 0.6 mg. of glucose, so that the $p_1 - p_0$ readings observed were threefold those indicated in Fig. 2, for 0.2 mg. of glucose. Fig. 2 is drawn to represent pressure change per 0.2 mg. of glucose to facilitate comparison with the blood analysis curves in Figs. 3 and 5. The latter are from analyses of normal blood, and the portions, equivalent to 0.2 cc. of blood, used for gasometric determination contain reducing substances equivalent to about 0.2 mg. of glucose.

Reduction by Blood Filtrate—1.5 cc. of each ferricyanide reagent was mixed with 3 cc. of a tungstic acid filtrate from normal blood, and determinations were made, as described above, with varying heating periods. The results are given in Table IV and Fig. 3.

TABLE V.

Gasometric Determination of Blood with Reagent Containing per Liter 8 Gm. of $K_3Fe(CN)_6$, 5 Gm. of Na_2CO_3 . Comparison with Folin-Wu and Shaffer-Somogyi Methods.

Blood No.	Mg. sugar found per 100 cc. blood.		
	Folin-Wu.	Shaffer-Somogyi.	Gasometric.
1	95.0	84.0	39.0
2	133.0	122.0	137.0
3	91.0	86.0	81.0
4	116.0	112.0	116.0

We desired a reagent which would give blood sugar values similar to those yielded by the Folin-Wu (1919), Shaffer-Somogyi (Somogyi, 1926), Hagedorn-Jensen (1923), and other standard methods in common use, which indicate average total blood sugar of about 100 mg. per cent in normal human blood. From the results in Figs. 2 and 3 and in Table IV it appeared that both Reagents 1 and 3 met this requirement. Reagents 2 and 4 show in Table IV evidence of too high reduction, due to non-glucose substances. The curve of Reagent 5 is too slow in approaching a horizontal direction. Reagent 1, with 0.5 per cent of Na_2CO_3 , gave in the filtrate-reagent mixture used about the same $NaCO_3$ concentration that obtains in the Hagedorn-Jensen (1923) filtrate-reagent mixture. It also gave results agreeing with the Folin-Wu, and practically completed its reaction with both glucose

and blood filtrate in 10 minutes. However, as shown in Table V, an occasional tungstic acid blood filtrate (Blood 1, Table V) has too much acidity to be neutralized by the low concentration of carbonate present in this reagent; in consequence of insufficient alkalinity in such a case reduction is incomplete and the sugar is found too low.

Ferricyanide Reagent 3 (Table IV), containing per liter 20 gm. each of sodium carbonate and bicarbonate, as is seen from Figs. 2 and 3 and Table IV, gives in 15 minutes more pressure per mg. of sugar than Reagent 1, gives only a slightly higher pressure after 30 minutes than after 15 minutes heating, and yields blood sugar values like those found by the Folin-Wu method. Comparison in analyses of normal and hyperglycemic bloods showed that with this reagent results agreed consistently with those by the Folin-Wu and Shaffer-Somogyi methods, and were unaffected by oxalate and fluoride in such amounts as are used to prevent coagulation of blood. It was apparent that a reagent heavily buffered with Na_2CO_3 and NaHCO_3 present in a ratio 1:1 by weight, offered optimum conditions.

The amounts of sodium carbonate and bicarbonate in the ferricyanide reagent were increased to 75 gm. each per liter in order to increase the factor of safety in buffer power, and to diminish the solubility of air in the solution obtained when the reagent was mixed with blood filtrate. The resulting reagent was found to give the same results as Reagent 3 in Table IV with respect to the amount of ferricyanide reduced per mg. of sugar.

Potassium carbonate and bicarbonate dissolve more readily than the sodium salts. For convenience in preparing solutions we therefore substituted 75 gm. each of K_2CO_3 and KHCO_3 per liter for the sodium salts, without altering the results. These concentrations of KHCO_3 and K_2CO_3 were thenceforward used in all the ferricyanide reagent solutions; only the ferricyanide was varied, in accordance with the amount of glucose encountered in each type of analysis.

Time Curves of Reduction of Ferricyanide by Glucose under Conditions of Blood and Urine Analyses.

In order to determine the rate and extent to which glucose reduces ferricyanide in these different reagents, the latter were heated

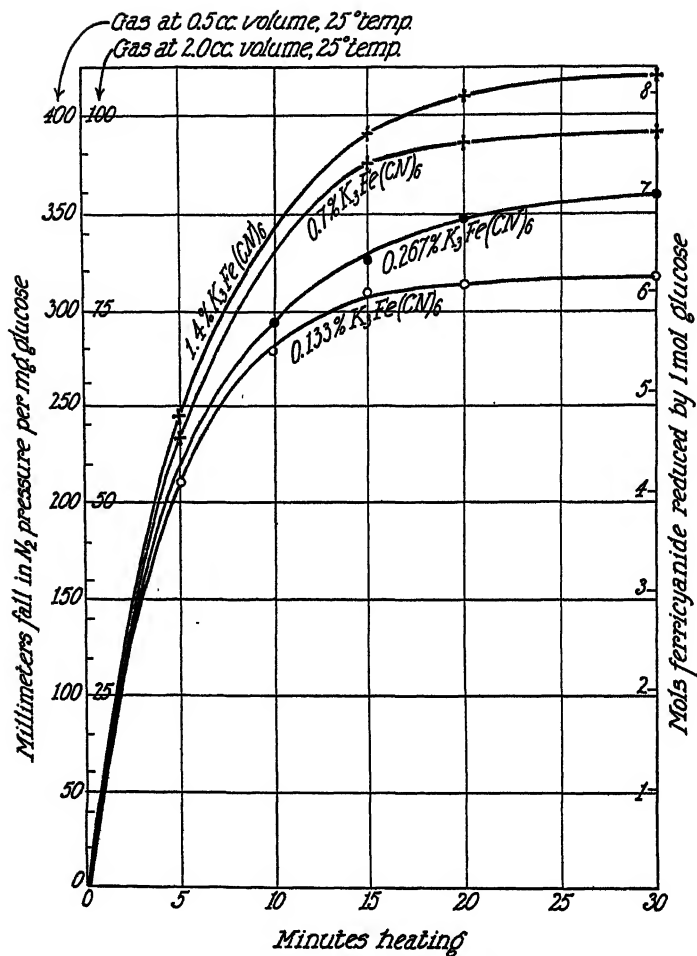


FIG. 4. Time curves of reduction of the ferricyanide of the different reagents by glucose.

+, ferricyanide reagent for urine. Contains 2.8 gm. $K_3Fe(CN)_6$ per 100 cc. Diluted to 1.4 gm. by mixture with 1 volume of glucose solution.

×, ferricyanide reagent with 1.4 gm. $K_3Fe(CN)_6$ per 100 cc. Diluted to 0.7 gm. by mixture with 1 volume of glucose solution.

●, ferricyanide reagent for macro blood sugar determination. Contains 0.8 gm. of $K_3Fe(CN)_6$ per 100 cc. Diluted to 0.267 by mixture with 2 volumes of blood filtrate.

○, ferricyanide reagent for micro blood sugar determination. Contains 0.4 gm. of $K_3Fe(CN)_6$ per 100 cc. Diluted to 0.133 by mixture with 2 volumes of blood filtrate.

Each reagent solution contained, in addition to the ferricyanide, 7.5 gm. each of K_2CO_3 and $KHCO_3$ per liter.

TABLE VI.

Determination of N₂ Pressure Fall per Milligram of Glucose under Conditions of Macro Blood Determination.

Reagent contains per liter 8 gm. of K₃Fe(CN)₆, 75 gm. of KHCO₃, 75 gm. of K₂CO₃.

1.5 cc. of reagent + 3.0 cc. of glucose solution heated. 3 cc. of mixture for gasometric determination. Gas pressure measured at 0.5 cc. volume.

Glucose concentration in standard solution.	Glucose represented in portion of solution used for gasometric determination.	N ₂ pressure fall observed $p_0 - p_1$.	Temperature of observation.	N ₂ pressure fall calculated for 25°.	N ₂ pressure fall per mg. of glucose at 25°.
15 min. heating period.					
0.3	0.60	190.3	22.0	192.4	321
		194.5	23.0	195.8	326
0.2	0.40	130.5	23.0	131.4	328
		128.0	19.5	130.5	326
0.1	0.20	63.6	21.7	64.3	321
		64.7	21.7	65.4	327
		64.4	21.3	65.2	326
		65.4	24.0	65.6	328
		65.0	24.0	65.2	326
		64.2	21.5	64.9	324
		64.0	20.0	65.1	325
		65.8	25.0	65.8	329
		64.7	23.0	65.2	326
		64.2	22.0	64.8	324
		64.6	22.0	65.2	326
0.06	0.12	38.2	23.0	38.4	321
0.05	0.10	32.7	25.0	32.7	327
		33.0	23.0	33.1	331
0.02	0.04	13.0	22.0	13.1	328
		12.6	21.0	12.8	320

TABLE VI—*Concluded.*

Glucose concentration in standard solution.	Glucose represented in portion of solution used for gasometric determination.	N ₂ pressure fall observed $p_0 - p_1$.	Temperature of observation.	N ₂ pressure fall calculated for 25°.	N ₂ pressure fall per mg. of glucose at 25°.
20 min. heating period.					
mg. per cc.	mg.	mm.	°C.	mm.	mm.
0.15	0.30	104.9	28.4	349.7	346
		104.8	28.4	349.3	346
		104.7	28.4	349.0	345
0.10	0.20	71.5	28.3	357.5	353
		70.1	28.3	350.5	347
		71.2	28.4	356.0	352
0.05	0.10	35.7	28.1	357.0	353
		35.6	28.1	356.0	352
		35.1	28.2	351.0	347

with standard glucose solutions under the conditions described above for blood and urine analyses, except that the duration of heating was varied. The blood reagents were each mixed with 2 volumes of 0.015 per cent glucose solution. The two reagents containing 2.8 and 1.4 gm. of ferricyanide per 100 cc. were mixed with 1 volume of 0.08 per cent and 1 volume of 0.1 per cent glucose solutions respectively; the conditions in the latter case were those for analysis of urine containing 2 per cent of glucose.

The results are shown in Fig. 4. They indicate that in all cases the reduction has become so nearly completed in 20 minutes that the ferricyanide decrease in the 20 to 30 minute interval is at the rate per minute of 1 part or less in 500. Consequently variations of a minute or two in the prescribed 20 minutes heating period do not introduce significant errors.

The number of mols of ferricyanide reduced by 1 mol of glucose increases with the concentration of ferricyanide present, but not in proportion to it. A 1000 per cent increase in the ferricyanide concentration, exemplified by the difference between the top and bottom curves, increases by only about 30 per cent the amount of ferricyanide reduced per mol of glucose.

Proportionality between Glucose Present and Ferricyanide Reduced.

For a definite time interval of heating, either 15 or 20 minutes, the results in Tables VI and VII indicate that, within the range of glucose concentrations for which each reagent is designed, the

TABLE VII.

Determination of N₂ Pressure Fall per Milligram of Glucose under Conditions of Urine Analysis.

Ferricyanide reagent contains per liter 28 gm. of K₃Fe(CN)₆, 75 gm. of KHCO₃, 75 gm. of K₂CO₃.

2 cc. of reagent and 2 cc. of glucose solution heated 20 minutes.

Glucose concentration of solution.	Glucose represented in portion of solution used for gasometric determination.	N ₂ pressure fall observed $p_0 - p_1$.	Temperature of observation.	N ₂ pressure fall calculated for 25°.	N ₂ pressure fall per mg. of glucose at 25°.
mg. per cc.	mg.	mm.	°C.	mm.	mm.
1.500	2.25	228.0	23.2	228.6	101.6
1.500	2.25	228.0	23.0	230.0	102.5
1.500	2.25	229.5	24.0	230.0	102.5
0.750	1.125	115.2	23.0	115.8	103.0
0.750	1.125	116.2	23.0	116.9	103.8
0.750	1.125	116.8	24.0	117.3	104.2
0.500	0.750	75.5	23.0	76.0	101.4
0.500	0.750	77.5	24.0	77.8	103.6
0.375	0.562	58.0	23.0	58.4	103.8
0.375	0.562	58.0	23.0	58.4	103.8
0.375	0.562	57.6	23.5	58.1	103.0
0.300	0.450	45.3	23.0	45.6	101.5
0.300	0.450	45.5	24.0	46.7	103.6
Average.....					103.0

amount of ferricyanide reduced is directly proportional to the glucose present: the amount of reduction per mg. of glucose, indicated by the fall in N₂ pressure at 25°, is constant for each ferricyanide reagent. This simple direct proportionality obviates the

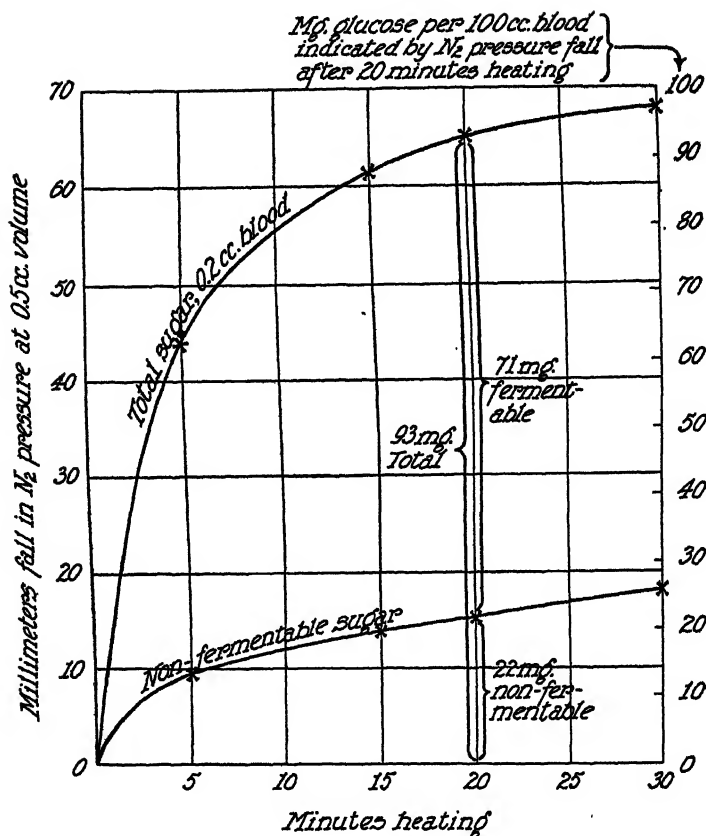


FIG. 5. Time curves of reduction, by blood filtrate, of ferricyanide reagent used for macro blood sugar determinations. The curves indicate amounts of ferricyanide reduced by "total sugar" present in 0.2 cc. of normal blood before treatment with yeast, and "unfermentable sugar" present after treatment with washed yeast by the procedure of Somogyi (1927). The N₂ pressure changes are measured at 25° temperature and 0.5 cc. volume.

necessity for factors varying with the amount of sugar present, and simplifies analytical calculations.⁴

⁴ The anhydrous glucose used to determine the factors was purified by recrystallization from alcohol. The specific rotation $[\alpha]_D^{20}$ was 52.2°. Theoretical specific rotation should be 52.6°, so that the glucose was 99.4 per cent pure. Dr. P. A. Levene kindly gave us this anhydrous glucose.

Time Curves of Reaction of Ferricyanide with Fermentable and Non-Fermentable Reducing Substances of Human Blood.

The results in Fig. 5 exemplify the relative amounts of fermentable and non-fermentable sugar found in normal human blood by our method, and the ratio at which they reduce ferricyanide. The determination of total blood sugar was carried out as described

TABLE VIII.
Recovery of Glucose Added to Blood.

Blood No.	Mixture analyzed.		Sugar found.	Observed increase due to added glucose (a).	Calculated increase due to added glucose (b).	Observed increase — calculated increase (a) — (b).
	Blood.	Glucose added.				
	cc.	mg.	mg. per 100 cc. blood	mg. per 100 cc. blood	mg. per 100 cc. blood	mg. per 100 cc. blood
1*	5	0	35		0	
1	5	15.0	335	300	300	0
2	5	0	118			
2	5	6.0	236	118	120	-2
3*	5	0	11		0	
3	50	50.0	110	99	100	-1
3	50	12.5	38	27	25	+2
3	50	5.0	22	11	10	+1
4	5	0	77		0	
4	50	100.0	277	200	200	0
4	20	20.0	175	98	100	-2
4	20	10.0	129	52	50	+2
4	20	5.0	101	24	25	-1

* Fermentable sugar had been removed by spontaneous glycolysis, by incubating blood at 38° for 24 hours.

above for the macro method. The non-fermentable sugar was determined by the same procedure, except that the fermentable sugar was removed by treatment of the blood with washed yeast according to Somogyi (1927) before the proteins were precipitated.

Recovery of Glucose Added to Blood.

Tests were made to see whether glucose added to blood was recovered. The results, given in Table VIII, show that all the added sugar is recovered.

Comparison with the Shaffer-Somogyi and Folin (1926) Blood Sugar Methods.

Comparison of results obtained by the Folin (1926) method, and Somogyi's (1926) modification of the Shaffer-Hartmann copper reduction method, with those yielded by the present gasometric macro blood sugar method in analyses of 50 bloods, normal and

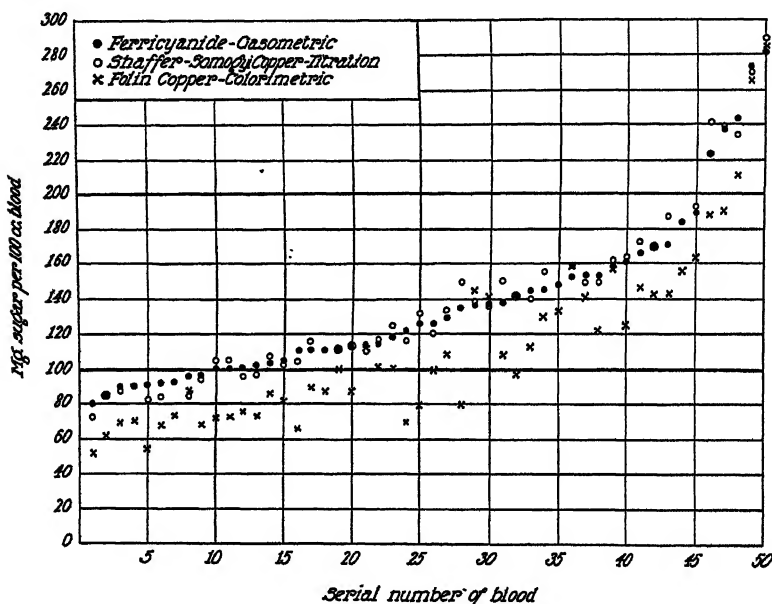


FIG. 6. Comparison of sugar found in blood by Folin copper-colorimetric, Shaffer-Somogyi copper-titration, and the gasometric methods. Ordinates represent mg. of sugar per 100 cc. of blood. Each symbol represents average of duplicate determinations.

pathological, is shown in Fig. 6. The gasometric method and Somogyi's modification of the Shaffer-Hartmann method agreed, usually within a few mg. per 100 cc. The Folin (1926) method, which was designed to give blood sugar values lower and nearer the true glucose content than the Folin-Wu and other previous methods, gave values which averaged about 20 mg. lower than the Shaffer-Somogyi and gasometric methods, but showed occasional peculiar

deviations, five of the 50 Folin values being equal to or higher than the values by the other two methods, while some of the Folin sugar values were as much as 60 mg. lower.

Comparison of Micro Gasometric Method with Shaffer-Somogyi and Macro Gasometric Methods.

Results obtained with the micro gasometric method compared with those obtained by the Shaffer-Somogyi and macro gasometric methods are given in Table IX.

TABLE IX.

Comparison of Micro Gasometric Method with Macro Gasometric and Shaffer-Somogyi Methods for Blood Sugar.

Blood No.	Mg. sugar per 100 cc. blood.		
	Shaffer-Somogyi.	Macro gasometric.	Micro gasometric.
1	114	113	116
2	194	190	191
3	242	224	230
4	120	126	114
5	84	92	84
6	72	80	76
7	116	111	112
8	165	162	160
9	240	239	246
10	132	126	126
11	150	136	136
12	136	138	136
13	162	159	165
14	159	153	151
15	171	171	165
16	179	180	180
17	107	103	99
18	97	102	99

Recovery of Glucose Added to Normal and Albuminous Urines.

In Table X are given the results of analyses of albuminous urines to which known amounts of glucose were added. In one of the albuminous urines (Urine 1, Table X) the albumin was removed by

Lloyd's reagent, while the other was prepared for analysis by mere dilution. The results show that the added glucose is recovered whether the urinary albumin is removed or not. Both

TABLE X.

*Recovery of Glucose Added to Urine Containing 0.12 Per Cent Albumin.
Comparison of Gasometric and Benedict's Methods.*

Urine No.	Glucose added.	Sugar found.		Increase found due to added glucose. Gasometric.	Error. Gasometric.
		Benedict.	Gasometric.		
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1	0.00	?	0.59		
1	1.80	2.30	2.39	1.80	0.00
1	1.35	1.84	1.96	1.37	+0.02
1	0.90	1.41	1.50	0.91	+0.01
2	0.00	?	0.42		
2	1.80	2.15	2.22	1.80	0.00
2	1.35	1.69	1.73	1.36	+0.01
2	0.90	1.28	1.32	0.90	0.00

Urine 1 contained 0.50 per cent fermentable sugar. Urine 2 contained 0.28 per cent fermentable sugar.

TABLE XI.

Comparison of Gasometric and Benedict's Quantitative Methods for Sugar in Diabetic Urine.

Diabetic Urine No.	Per cent sugar.	
	Benedict.	Gasometric.
1	1.10	1.15
2	1.90	1.90
3	1.92	1.90
4	0.60	0.51
5	0.80	0.74
6	0.50	0.50

these urines were taken from patients with degenerative nephritis, in order to obtain albuminous specimens. The high content of reducing material before addition of glucose is due to fermentable sugar; true glycosurias are frequent in such urines.

Comparison of Sugar in Diabetic Urine by Benedict Titration and Present Gasometric Methods.

Comparison of results obtained by the Benedict (1911) and the present gasometric urine sugar methods is given in Table XI. The two methods agreed within 0.1 per cent.

SUMMARY.

A gasometric method is described for determining reducing sugars. The latter are permitted to react with ferricyanide, of which an amount proportional to the sugar is reduced to ferrocyanide. The decrease in ferricyanide caused by the sugar is measured by the decrease in the pressure of N_2 gas observed when the ferricyanide liberates N_2 from hydrazine in the Van Slyke-Neill manometric apparatus.

Applications to rapid determination of sugar in blood and urine are described.

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STUDIES OF GAS AND ELECTROLYTE EQUILIBRIA IN BLOOD.

XIV. THE AMOUNTS OF ALKALI BOUND BY SERUM ALBUMIN AND GLOBULIN.

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In this paper data are presented concerning the alkali bound by serum albumin and serum globulin at reactions between pH 6.8 and 7.8 at 38° in solutions of physiologically normal salt content. These data have been used to calculate the base bound by the total protein of serum, and the amount so calculated has been compared with observed values.

Van Slyke, Wu, and McLean (1) have presented similar data for the total proteins of horse serum. Such data do not, however, apply accurately to normal human serum, in which the albumin:globulin ratio is much higher. Furthermore, in pathological human serum, the content of total proteins and likewise the albumin:globulin ratio may differ greatly from these values in normal serum. In order to estimate accurately the base bound by the serum proteins it is necessary to know the base-binding capacity of the albumin and of the globulin, and the amount of each present.

Cohn (2) in his thorough review of the physical chemistry of proteins has collected titration curves of serum albumin from many sources, which show substantial agreement. The titration curves of serum globulin show much less agreement, however. The interest of previous investigators in the titration curves has been to determine the characteristics of the purified protein over a wide pH range. Our interest has been in accurate determination

of the base bound by the proteins over the narrow pH range of the circulating blood plasma and under physiological conditions of temperature and salt concentration.

EXPERIMENTAL.

Preparation of Serum Globulin and Albumin.—The globulins were salted out of serum by means of half saturation with ammonium sulfate. After filtration the globulins were redissolved and salted out again. This process was repeated four or five times until the amount of nitrogen per cc. of filtrate, other than ammonia nitrogen, became constant. The globulins were again put into solution and dialyzed in collodion bags until the ammonium sulfate was removed. The solution was concentrated by suspending the collodion bags in the breeze of an electric fan, and was finally subjected to pressure dialysis against distilled water which was saturated with CO_2 to bring its reaction near the isoelectric point of serum globulin.

The serum albumin was prepared from the filtrate from the globulin by saturation with ammonium sulfate. After filtration the albumin was put into solution, the amount of ammonia was estimated, and adherent globulins were removed by addition of sufficient ammonium sulfate to make a half saturated solution. Albumin was again salted out by saturation with ammonium sulfate. This process was continued as long as globulins were found present. The albumin solution was then dialyzed in the same manner as the globulin solution.

Preparation of Solutions.—To prepare as concentrated solutions of the proteins as possible, measured amounts of NaCl and NaOH were added directly to the dialyzed albumin solutions or globulin suspensions.

Determination of Base Bound by Proteins.

A. *Titration with CO_2 .*—The principle has been used in previous papers of this series (3, 4). The protein is dissolved with NaOH, and the solution is saturated with an inert gas (hydrogen in the present case) containing varying proportions of CO_2 . The NaOH is entirely converted into proteinate, NaP , and bicarbonate,

NaHCO_3 . (The proportions of Na_2CO_3 under our conditions are negligible.) Hence the NaP is calculated as

$$(1) \quad [\text{NaP}] = [\text{NaOH}] \text{ added} - [\text{NaHCO}_3]$$

The NaHCO_3 is calculated as

$$(2) \quad [\text{NaHCO}_3] = \text{total } [\text{CO}_2] - [\text{H}_2\text{CO}_3]$$

The concentrations have all been estimated as millimols or milli-equivalents per kilo of water in the solutions.

The total CO_2 contents of the solutions were determined by gasometric analysis (5).

$[\text{H}_2\text{CO}_3]$, representing physically dissolved CO_2 in mm per kilo of water, was calculated from the CO_2 tension, p_{CO_2} , at 38° , as

$$(3) \quad \text{mm } [\text{H}_2\text{CO}_3] = \frac{1000 \alpha^0 \text{CO}_2}{22.26} \times \frac{p_{\text{CO}_2}}{760}$$

The value α^0 , indicating cc. of CO_2 dissolved per gram of water at 760 mm. CO_2 tension, was found, in a preceding paper of this series (6), to be reduced by salts, and to be relatively unaffected by serum proteins. Our serum protein solutions contained Na salts in a total concentration of 136 to 138 milli-equivalents per kilo of water; of these salts about 105 milli-equivalents were in the form of NaCl. We can estimate the depression of CO_2 solubility caused by the Na salts with negligible error if we assume that the depression of α^0 below α^0 of pure water is equal to the depression caused by the presence of 137 millimols of NaCl per kilo of water. From Table VI of Van Slyke, Sendroy, Hastings, and Neill (6) this depression is found to be 0.014. The α^0 value of pure water, by the same authors, being 0.547, the α^0 value of our protein solutions is estimated as

$$(4) \quad \alpha^0 = 0.547 - 0.014 = 0.533$$

Hence the $[\text{H}_2\text{CO}_3]$ of our protein solutions is calculated as

$$(5) \quad \text{mm } [\text{H}_2\text{CO}_3] \text{ per kilo H}_2\text{O} = 0.0315 p_{\text{CO}_2}$$

The saturation of the solutions and the subsequent separation of the gas and liquid phases were carried out according to the "First Procedure" described in Paper I of this series (7).

The CO_2 content of the gas phase was determined by Y. Henderson's (8) modification of the Haldane apparatus. From the percentage CO_2 the CO_2 tension was calculated by the usual formula

$$(6) \quad p_{\text{CO}_2} = \frac{(\text{per cent } \text{CO}_2 \text{ in gas}) \times (\text{barometric pressure} - 49.7)}{100}$$

49.7 mm. of mercury being the vapor tension of water at 38° .

The total CO_2 content of the liquid phase was determined with the manometric blood gas apparatus of Van Slyke and Neill (5), with the factors of Van Slyke and Sendroy (9) for calculating the results.

H_2O content was determined by drying 2 cc. portions of the solutions to constant weight at 105° .

Total nitrogen was determined by Kjeldahl analyses.

Total base was determined according to Fiske's (10) principle, by transforming the alkali salts into sulfates, and estimating the SO_4 by benzidine titration. The procedure was that described by Van Slyke, Hiller, and Berthelsen (11) on p. 671 of their paper. Their gasometric method had not been perfected at the time of the experiments here reported.

Electrometric pH was determined at 38° in the Clark-Cullen electrode vessel (12). The $\text{H}_2\text{-CO}_2$ gas mixture used in the electrode was the same with which the solution had been equilibrated prior to determination of its total CO_2 content.

Electrometric pH was calculated, as in previous papers of this series (13), by the usual formula for 38° ,

$$\text{pH} = \frac{E - E_0}{0.06169}$$

The value of E_0 was determined daily by standardizing the saturated calomel cell against 0.1 N HCl, which was assumed to have a pH of 1.08. All potential measurements were corrected to 760 mm. of hydrogen pressure.

Gasometric pH values, as checks on the electrometric pH determinations, were calculated by Hasselbalch's equation,

$$(7) \quad \text{pH} = \text{pK}' + \log \frac{[\text{BHCO}_3]}{[\text{H}_2\text{CO}_3]}$$

The value taken for pK' in these solutions was 6.11.

B. Titration with HCl.—The proteins were dissolved in enough NaOH to render their solutions distinctly alkaline, and were titrated electrometrically with 0.1 N HCl in the titration vessel of Hastings (14). An improved form of the vessel, previously described (15), was used.

The base bound by the protein was found by subtracting the HCl added from the NaOH originally placed in the solution. In the pH range covered by these titrations, all the Na is in the forms NaP and NaCl, NaOH being negligible.

Results.

The results obtained by titrating two preparations of serum albumin with CO₂ are given in Tables I and II. The results of an electrometric titration are given in Table III. The average straight line through the points plotted according to these results in Fig. 1 is represented by the equation,

$$(8) \quad B \text{ (Alb.)} = 0.78 \text{ (Alb. N)} (\text{pH} - 5.16)$$

where B (Alb.) represents milli-equivalents of base bound by albumin, and (Alb. N) represents grams of albumin nitrogen. Similarly the results with globulin from Tables IV, V, and VI, plotted in the lower curve of Fig. 1, are represented by the equation,

$$(9) \quad B \text{ (Gl.)} = 0.48 \text{ (Gl. N)} (\text{pH} - 4.89)$$

These simple linear equations are valid only for the limited pH range from 6.8 to 7.8 covered by the experiments. There is no reason to assume that the curves representing the relationship of base bound by albumin or globulin to the pH of their solutions should be linear. They happen, over the limited range of physiological pH, to approximate straight lines with sufficient closeness to enable us to represent the results by linear formulæ with errors no greater than those of the experimental measurements.

The total amount of base bound by both proteins, or protein groups, in serum is calculated by the combined equation,

$$(10) \quad \text{BP.} = 0.78 \text{ (Alb. N)} (\text{pH} - 5.16) + 0.48 \text{ (Gl. N)} (\text{pH} - 4.89)$$

which represents the sum of the amounts of base bound by the albumin and globulin respectively.

The applicability to human serum of this equation, based on data obtained from horse proteins, was tested as follows. A sample of mixed

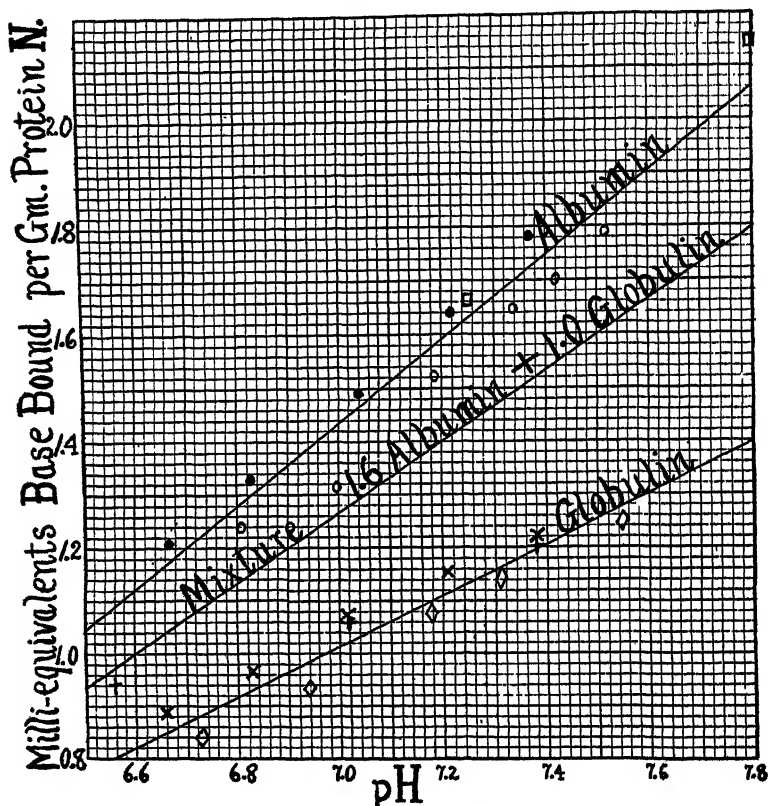


FIG. 1. The points represent experimental results from the tables as follows: ●, Table I, CO_2 titration of albumin. ○, Table II, CO_2 titration of albumin. □, Table III, electrometric titration of albumin. ×, Table IV, CO_2 titration of globulin. +, Table V, electrometric titration of globulin. ◇, Table VI, electrometric titration of globulin.

The linear curves indicate the mean values of results from the different albumin and globulin preparations, and the estimated values for a mixture of 1.6 parts of albumin and 1.0 part of globulin, such as is approximated in normal human serum.

human serum was dialyzed in collodion bags against distilled water saturated with CO_2 for 3 months at 0° . It was then made up into a solution containing per liter 100 millimols of NaCl and 30 milli-equivalents of NaOH .

TABLE I.

Base Bound by Serum Albumin, Determined by Titration with CO₂.

Preparation 1.

Protein N = 7.56 gm. per kilo H₂O.
 NaCl added = 104.6 mm " " "
 NaOH " = 31.38 " " " "
 Total Na " = 136.0 " " " "
 H₂O = 0.956 gm. per cc.

Experi- ment No.	CO ₂ tension.	[H ₂ CO ₃]	Total [CO ₂].	[BHCO ₃]	[BP] + [BHCO ₃]	[BP]	[BP]	pH electro- metric.	pH cal- culated as 6.11 + log [BHCO ₃] [H ₂ CO ₃].
	mm.	mm per kg. H ₂ O	mm per kg. H ₂ O	mm per kg. H ₂ O	m.-eq. per kg. H ₂ O	m.-eq. per kg. H ₂ O	m.-eq. per gm. N		
1	205.8	6.48	28.76	22.28	31.38	9.10	1.204	6.67	6.65
2	128.8	4.06	25.40	21.34	31.38	10.04	1.327	6.83	6.83
3	75.7	2.38	22.46	20.08	31.38	11.30	1.494	7.04	7.04
4	46.4	1.462	20.42	18.96	31.38	12.42	1.642	7.22	7.23
5	30.6	0.964	18.85	17.89	31.38	13.49	1.784	7.37	7.38

TABLE II.

Base Bound by Serum Albumin, Determined by Titration with CO₂.

Preparation 2.

Protein N = 8.93 gm. per kilo H₂O.
 NaCl added = 105.2 mm " " "
 NaOH " = 31.57 " " " "
 Total Na " = 136.8 " " " "
 H₂O = 0.951 gm. per cc.

Experi- ment No.	CO ₂ tension.	[H ₂ CO ₃]	Total [CO ₂].	[BHCO ₃]	[BP] + [BHCO ₃]	[BP]	[BP]	pH electro- metric.	pH cal- culated as 6.11 + log [BHCO ₃] [H ₂ CO ₃].
	mm.	mm per kg. H ₂ O	mm per kg. H ₂ O	mm per kg. H ₂ O	m.-eq. per kg. H ₂ O	m.-eq. per kg. H ₂ O	m.-eq. per gm. N		
1	128.7	4.05	24.56	20.51	31.57	11.06	1.238	6.81	6.81
2	78.0	2.46	22.28	19.82	31.57	11.75	1.315	7.00	7.02
3	47.0	1.48	19.41	17.93	31.57	13.64	1.526	7.19	7.19
4	31.6	0.996	17.84	16.84	31.57	14.73	1.648	7.34	7.34
5	24.0	0.756	17.01	16.25	31.57	15.22	1.703	7.42	7.44
6	18.5	0.583	16.15	16.57	31.57	16.00	1.790	7.52	7.56

Portions of the solution were equilibrated at 38° with atmospheres containing varying tensions of CO₂ and analyzed as in the previous experi-

TABLE III.

Base Bound by Serum Albumin, Determined by Electrometric Titration with 0.1 N HCl.

Preparation 1. Protein N = 7.56 gm. per kilo H₂O.

NaCl added = 104.6 mm " " "

NaOH " = 31.38 " " " "

Total Na " = 136.0 " " " "

H₂O = 0.9563 gm. per cc.

Experiment No.	[HCl]	[BP + BCl]	[BP]	[BP]	pH electro-metric.
	<i>m.-eq. per kg. H₂O</i>	<i>m.-eq. per kg. H₂O</i>	<i>m.-eq. per kg. H₂O</i>	<i>m.-eq. per gm. N</i>	
1	15.15	31.38	16.23	2.15	7.80
2	18.50	31.38	12.88	1.70	7.25
3	22.6	31.38	8.78	1.16	6.37
4	25.8	31.38	5.58	0.74	5.70
5	29.7	31.38	1.68	0.22	5.20

TABLE IV. .

Base Bound by Serum Globulin, Determined by Titration with CO₂.

Preparation 1. Protein N = 11.31 gm. per kilo H₂O.

NaCl added = 106.7 mm " " "

NaOH " = 32.0 " " " "

Total Na " = 138.7 " " " "

H₂O = 0.939 gm. per cc.

Experiment No.	CO ₂ tension.	[H ₂ CO ₃]	Total [CO ₃]	[BHCO ₃]	[BP] + [BHCO ₃]	[BP]	[BP]	pH electro-metric.	pH calculated as $6.11 + \log \frac{[BP]}{[H_2CO_3]}$
	<i>mm.</i>	<i>mm per kg. H₂O</i>	<i>mm per kg. H₂O</i>	<i>mm per kg. H₂O</i>	<i>m.-eq. per kg. H₂O</i>	<i>m.-eq. per kg. H₂O</i>	<i>m.-eq. per gm. N</i>		
1	206.5	6.50	28.49	21.99	32.00	10.01	0.885	6.66	6.64
2	132.2	4.16	25.25	21.09	32.00	10.91	0.965	6.83	6.82
3	80.8	2.55	22.44	19.89	32.00	12.11	1.075	7.02	7.00
4	48.7	1.534	20.47	18.94	32.00	13.06	1.156	7.21	7.20
5	29.8	0.939	19.16	18.22	32.00	13.78	1.219	7.38	7.40

ments. The values for base calculated by the above formula to be bound by the albumin and globulin present at the observed pH points are given in the column headed "BP calculated" (Table VII).

It is evident from the last column of Table VII, showing the ratio of observed to calculated BP values, that the equation has

TABLE V.

Base Bound by Serum Globulin, Determined by Electrometric Titration with 0.1 N HCl.

Protein N = 11.31 gm. per kilo H₂O.
NaCl added = 106.7 mm " " "
NaOH " = 32.0 " " " "
Total Na " = 138.7 " " " "
H₂O = 0.938 gm. per cc.

Experiment No.	[HCl]	[BP + BCl]	[BP]	[BP]	pH electro-metric.
	<i>m.-eq. per kg. H₂O</i>	<i>m.-eq. per kg. H₂O</i>	<i>m.-eq. per kg. H₂O</i>	<i>m.-eq. per gm. N</i>	
1	12.06	32.00	19.94	1.76	9.26
2	18.45	32.00	13.55	1.20	7.38
3	19.95	32.00	12.05	1.06	7.02
4	21.41	32.00	10.59	0.94	6.56

TABLE VI.

Base Bound by Serum Globulin, Determined by Electrometric Titration with 0.1 N HCl.

Total N = 5.80 gm. per kilo H₂O.
Total NaCl added = 103.7 mm " " "
" NaOH " = 31.12 " " " "
H₂O = 0.9640 gm. per cc.

Experiment No.	[HCl]	[BP + BCl]	[BP]	[BP]	pH electro-metric.
	<i>m.-eq. per kg. H₂O</i>	<i>m.-eq. per kg. H₂O</i>	<i>m.-eq. per kg. H₂O</i>	<i>m.-eq. per gm. N</i>	
1	21.60	31.12	9.52	1.64	8.74
2	23.84	31.12	7.28	1.25	7.55
3	24.47	31.12	6.65	1.15	7.31
4	24.85	31.12	6.27	1.08	7.18
5	25.70	31.12	5.42	0.935	6.94
6	26.22	31.12	4.90	0.844	6.73
7	27.55	31.12	3.57	0.615	6.40

indicated about 106 per cent of the base actually observed in combination with the proteins. Whether the 6 per cent difference indicates an actual species difference between the proteins of horse

and man, or whether the difference is due to summated experimental error, we cannot state. In view of the possibilities of error from traces of electrolytes adherent to the dialyzed proteins, and from uncertainty concerning the accuracy with which the Howe technique in the serum separates and determines the exact amounts of pure albumin and globulin present, we are inclined to believe that the difference may be due to such errors, and to use the formula for human serum as well as horse serum until titration curves on purified human proteins can be obtained.

TABLE VII.

Base Bound by Human Serum Proteins, Determined by Titration with CO₂.

Total protein N	=	5.06 gm. per kilo H ₂ O.
Albumin "	=	2.75 " " " "
Globulin "	=	2.31 " " " "
NaCl added	=	104.3 mm " " "
NaOH "	=	31.36 " " " "
Total Na	=	136.0 m.-eq. " " "
H ₂ O	=	0.958 gm. per cc.

Experiment No.	CO ₂ tension.	[H ₂ CO ₃]	Total [CO ₂]	[BHCO ₃]	[BP] + [BHCO ₃]	[BP] found.		pH calculated as 6.11 + log [BHCO ₃] [H ₂ CO ₃]	[BP] calculated by Equation 10.	[BP] found [BP] calculated
	mm.	mM per kg. H ₂ O	mM per kg. H ₂ O	mM per kg. H ₂ O	m.-eq. per kg. H ₂ O	m.-eq. per kg. H ₂ O	m.-eq. per gm. N		m.-eq. per kg. H ₂ O	
1	131.6	4.14	29.85	25.71	31.36	5.65	1.12	6.90	6.01	0.94
2	79.9	2.52	27.71	25.19	31.36	6.17	1.22	7.11	6.66	0.93
3	54.8	1.726	26.32	24.69	31.36	6.67	1.32	7.27	7.21	0.98
4	30.0	0.946	24.76	23.81	31.36	7.55	1.49	7.51	7.97	0.95

In normal human serum the ratio of albumin to globulin averages about 1.6 (16). With this ratio a combination of the two formulae gives, as the relation between total protein nitrogen and base bound by protein, what might be termed an average normal formula as follows:

$$(11) \quad BP_s = 0.66 \times \text{grams N} \times (pH_s - 5.08)$$

or

$$(12) \quad BP_s = 0.104 \times \text{grams protein} \times (pH_s - 5.08)$$

In normal horse serum we have found an albumin:globulin ratio of 0.8. As calculated by Equation 10 such a mixture binds per gram of nitrogen only 93 per cent as much alkali at pH 7.4 as does the albumin-globulin mixture usually approximated in normal human serum.

Albumin:globulin ratios even lower than 0.8 are encountered in human serum in some pathological conditions, notably nephrosis. In such sera the use of Equation 11 for normal serum might lead to as much as 10 per cent error in estimating the base bound by the total protein. For more accurate estimates of the BP, in such cases it is necessary to determine the albumin and globulin contents and estimate BP, by Equation 10.

For estimating the base bound by the total proteins of horse and human serum respectively two equations have been published in previous papers of this series (1, 17). Both yield considerably lower results than those which our present data indicate to be correct. The formula for horse serum previously published (1) indicated at pH 7.4 only 76 per cent as much base bound per gram of protein as is represented in Equation 10, for an albumin:globulin ratio of 0.8. The previously published formula for human serum (17) indicated at pH 7.4 85 per cent as much base as accords with present data for an albumin:globulin ratio of 1.6. It is probable that in the experiments on which the former equations were based dialysis was not pushed quite far enough to remove the last traces of preexisting base from the protein preparations used.

SUMMARY.

The relationship between pH and amount of alkali bound by the proteins has been determined for purified albumin and globulin of horse serum over the range of pH between 6.8 and 7.8.

Within these limits the relationships at 38° are expressed by the empirical linear equations:

$$\begin{array}{l} \text{M.-eq. base bound per gram albumin } N = 0.78 (\text{pH} - 5.16). \\ \text{“ “ “ “ “ globulin “} = 0.48 (\text{pH} - 4.89). \end{array}$$

The base bound by the proteins of human serum agreed, within 6 per cent, with the amounts calculated from the albumin and globulin contents, on the assumption that these proteins in human serum have the same alkali titration curves as in horse serum.

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STUDIES OF GAS AND ELECTROLYTE EQUILIBRIA IN BLOOD.

XV. LINE CHARTS FOR GRAPHIC CALCULATIONS BY THE HENDERSON-HASSELBALCH EQUATION, AND FOR CALCULATING PLASMA CARBON DIOXIDE CONTENT FROM WHOLE BLOOD CONTENT.

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1. *Graphic Calculations by the Henderson-Hasselbalch Equation.*

The Henderson equation

$$(1) \quad [H^+] = K' \frac{[H_2CO_3]}{[BHCO_3]}$$

in the logarithmic form

$$(2) \quad pH = pK' + \log \frac{[BHCO_3]}{[H_2CO_3]}$$

in which Hasselbalch (1917) used it, has found much application in the calculation of pH_s from known CO_2 content and CO_2 tension of blood, or of CO_2 tension from known pH_s and CO_2 content. For these purposes $[H_2CO_3]$ in millimols per liter is calculated from the CO_2 tension, p , and the solubility coefficient, α , as $0.0591 \alpha p$; $[BHCO_3]$ is estimated by subtracting this $[H_2CO_3]$ from the total CO_2 content, $[CO_2]$. The equation thence takes the form given in the third formula in Table IV of Paper I of this series (Austin, *et al.*, 1922); *viz.*

$$(3) \quad pH = pK' + \log \frac{[CO_2] - 0.0591 \alpha p}{0.0591 \alpha p}$$

For calculation of CO₂ tension the equation takes the form of the fifth formula in the table mentioned above.

$$(4) \quad p = \frac{[\text{CO}_2]}{0.0591 \alpha (10^{pK' - pH} + 1)}$$

The labor and chance of error involved in making the computations by means of Equations 3 and 4, and other equations derived from Henderson's, can be avoided by application of graphic methods for the solution of equations with three variables. Of such methods that of the d'Ocagne nomogram or line chart, introduced into physiology by Henderson (Henderson, Bock, Field, and Stoddard, 1924), appears the most suitable. In Fig. 1 such a line chart is given. A straight line drawn through given points on any two of the scales cuts the other scales at points indicating the values that would be calculated by the above equations. One can thus calculate any one of the variables represented from any two others. Twelve different calculations are thus possible. Two of them, the calculation of pH_s and CO₂ tension from [CO₂]_s and [BHCO₃]_s, are not practicable, because of the relatively large error caused in the value calculated by slight errors in [CO₂]_s or [BHCO₃]_s. The other ten calculations are, however, all practically applicable. The ones most used in the past literature are the following six:

pH_s, from *p* and [CO₂]_s, or from *p* and [BHCO₃]_s;

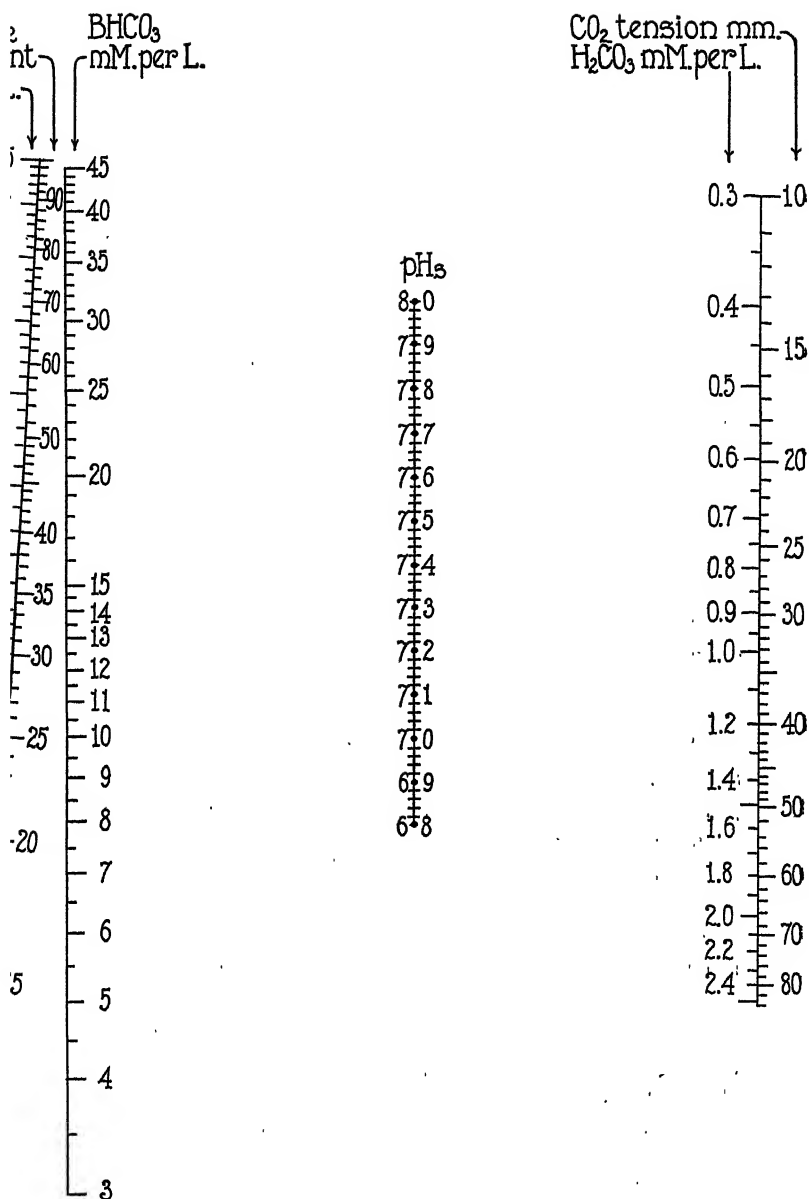
p, from pH_s and [CO₂]_s, or from pH_s and [BHCO₃]_s;

[BHCO₃]_s, from [CO₂]_s and pH_s, or from [CO₂]_s and *p*.

(The subscript *s* is used to indicate serum or plasma values.)

For the calculations involving only *p*, pH_s, and [BHCO₃]_s, Fig. 1 is theoretically exact. For calculations in which [CO₂]_s is involved the accuracy of the chart is approximate, but the degree of approximation is so close, for the range of values found in either normal or pathological blood, that the error of calculation is well within that of the experimental determinations involved.

The precision of the graphic, as well as the algebraic, application of the Henderson-Hasselbalch equation depends upon the accuracy of the constants, α and pK' . The value of the solubility coefficient, α , for serum has recently been ascertained by direct determination (Van Slyke, Sendroy, Hastings, and Neill, 1928) and found to be



Line chart for application of the Henderson-Hasselbalch equation to blood
A straight line intersecting two scales cuts the other scales at points which
imultaneously occurring values.

0.510. The value of pK' , as the result of recent determinations in several laboratories, summarized in a preceding paper (Hastings, Sendroy, and Van Slyke, 1928), has been found to be 6.10, probably with an error not exceeding ± 0.01 . The scales of Fig. 1 have been computed with these constants. For normal serum, and for plasma from blood with not over 0.2 per cent of potassium oxalate added, pK' and α appear to be constant within the limits of experimental determination. Even in pathological serum and plasma the variations are seldom sufficient to affect significantly results calculated by Equations 3 and 4 or by the line chart in Fig. 1.

Use of Fig. 1 for Estimating CO₂ Tension in Whole Blood.—When CO₂ content, hemoglobin content or cell volume, and pH_s of whole blood are known, the CO₂ content of the serum or plasma can be estimated with the help of Fig. 3. The values for serum CO₂ and pH are then applied to Fig. 1. The possible error is increased from about 1 mm. to about 3 mm. of CO₂ tension when plasma CO₂ values thus estimated from whole blood are used in place of those directly determined by plasma analysis.

It is possible to determine the pH of plasma in the whole blood by the electrometric methods, which, as shown by Parsons (1917) indicate plasma pH even when applied to whole blood. Hawkins (1923) has adapted Cullen's (1922) colorimetric method also to use with micro portions of whole blood.

The preliminary use of Fig. 3 in this case has the same effect as adding a correction to the pK' value of serum (Warburg, 1922; Van Slyke, Wu, and McLean, 1923) in order to make Hasselbalch's equation applicable to whole blood with its lower CO₂ content.

Construction of Fig. 1.—The $[BHCO_3]$, pH, and $[H_2CO_3]$ scales of Fig. 1 are constructed on the same principle as the familiar slide-rule, with which multiplication is performed by graphic addition of logarithms.

The equation

$$xy = z$$

can be written

$$\log x + \log y = \log z.$$

For illustration we draw the line chart shown in Fig. 2, with values of x and y between 1 and 10 at logarithmic intervals on the two outer lines. On the

inner z line, midway between the others we draw a logarithmic scale with intervals half as long as those of the outer scales, so that the inner line, in the same distance covered by a single logarithmic scale for numbers from 1 to 10 on each outer line, contains two similar scales, covering a total interval of values for numbers from 1 to 100.

We draw the line abc across the scales. It cuts the inner z scale at a height half as great above the 1 point as the height at which it cuts the y

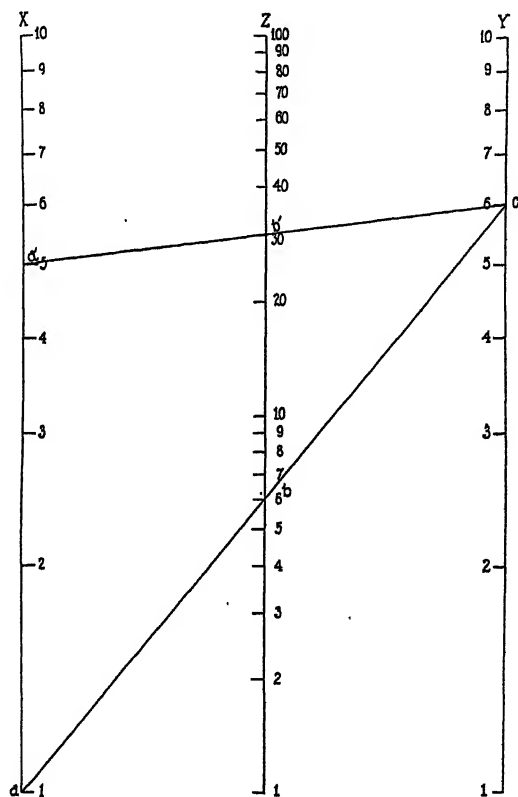


FIG. 2.

scale at c . But since the logarithmic scales on the z line are only half as long as on the y scale, the same value for z as for y is indicated. Since the value on the x scale is 1, the line abc indicates the equation, $xy = z$, when x is 1, and z therefore is equal to y .

If now we raise the cross line to the position $a'b'c$, we raise its intersection on the z line half as much as on the x line. But the raise covers the same portion of a logarithmic scale on both lines, since the z scales are half as long

as the x scales. We have therefore, while increasing $\log x$ from the value at a to that at a' , increased $\log z$ by the same amount. Hence z has been increased in direct proportion to x . And in general it is apparent that with a line chart constructed in this manner, any line cutting the scales indicates at the intersections x , y , and z values according with the equation, $xy = z$.

To make the chart hold for the equation, $xy = kz$, it is necessary only to change the level of the z scale so that a line connecting the points for x and y values of 1 cuts the z scale at k instead of 1.

For the equation $\frac{x}{y} = z$, the y scale is inverted so that it reads 10, 9, 8, etc., from the bottom upwards instead of from the top downwards: then the effect of an increase in $\log y$ is to cause an equal decrease in $\log z$.

The equation $\frac{x}{y} = kz$ is the form of the Henderson equation, which may be written $\frac{[\text{H}_2\text{CO}_3]}{[\text{BHCO}_3]} = \frac{1}{k} \times [\text{H}^+]$. The line chart is therefore theoretically exact for Equations 1 and 2.

Equations 3 and 4, in which the total CO₂ content instead of the bicarbonate is related to the CO₂ tension and the pH, cannot be cast into a form theoretically capable of exact solution by a logarithmic line chart. However, the scale of [CO₂] values on Fig. 1 was empirically located in such a manner that for values of pH_s, [CO₂], and p within the range found in normal or pathological human blood, this scale can be used without significant error. Points on the [CO₂] scale of Fig. 1 at brief intervals were located by calculating for each [CO₂] value three pairs of [BHCO₃] and [H₂CO₃] values. For this purpose three CO₂ tensions were taken, representing as nearly as could be judged from available data the extremes and mean that would be likely to accompany the given CO₂ value in human blood. The corresponding [BHCO₃] values were then calculated by the formula, [BHCO₃] = [CO₂] - [H₂CO₃], the [H₂CO₃] values being read from the inner side of the right hand scale of Fig. 1. Three lines were drawn across the chart, each intersecting the [BHCO₃] and [H₂CO₃] scales at the calculated points. If a theoretically exact scale for [CO₂] values were capable of location, these three lines would intersect upon it at a common point. In fact they do not. However, their intersections were so close together, that when the calculated [CO₂] point was located in the center of each triangle, of which the three intersections formed the vertices, the scale obtained by connecting [CO₂] points so located was sufficiently exact. In this manner the curved [CO₂] scale at the left of Fig. 1 was plotted. pH_s values estimated by means of this [CO₂] scale and the pCO₂ scale are within 0.005 of the pH_s values calculated algebraically by Equation 3.

2. Graphic Estimation of CO₂ Content of Blood Serum or Plasma from CO₂ Content of Whole Blood.

Since the CO₂ or BHCO₃ in a unit volume of blood cells may be but little more than half that in a unit volume of plasma from the

same blood, the CO_2 content of the whole blood is to a marked degree dependent upon the proportion of cells present. For this reason the CO_2 and bicarbonate contents of whole blood are less accurate indicators of the acid-base balance of the organism than values determined in serum or plasma. The bicarbonate content of anemic blood will be high even when the bicarbonate concentration in both cells and serum is that of average normal blood, and *vice versa* for polycythemic blood. In order to exclude the variable effect of cell content, it is therefore desirable to make determinations of bicarbonate or CO_2 content on plasma or serum when the values are to be used to estimate the state of the acid-base balance.

However, it is not always practicable to centrifuge a sample of blood and bring the plasma to analysis, with the maintenance throughout of precautions to prevent loss of CO_2 . In such cases it is desirable to be able to determine the CO_2 content in the whole blood, and estimate thence the content of the plasma.

Peters, Bulger, and Eisenman (1923-24) have published a formula and chart for such an estimation, but they are limited in application to oxygenated blood with known CO_2 tension.

The results of Van Slyke, Wu, and McLean (1923) indicate that definite relationships exist between the CO_2 contents of cells and serum, depending chiefly upon the pH, and the degree of oxygenation of the hemoglobin. It accordingly seemed possible to ascertain factors for calculating plasma and serum CO_2 from whole blood CO_2 which would be valid under all variations of reaction and oxygenation that can exist in the circulating blood. The theoretical equations expressing these relationships are too complicated for practical routine application. It has, however, proved possible to express the relationships with satisfactory precision in a line chart, which provides a simple means for graphic estimation of the CO_2 content of serum or plasma from that of whole blood, of any hemoglobin or cell volume content, at any degree of oxygenation, and at any pH, within the range of normal or pathological blood. This chart is given in Fig. 3.

Use of Fig. 3 to Determine Plasma CO_2 from Whole Blood CO_2 .— A straight line drawn from any pH_s point to the scale with cell volume and oxygen capacity values intersects the slanting *f* scale at a point indicating the factor, *f*, by which the CO_2 content of

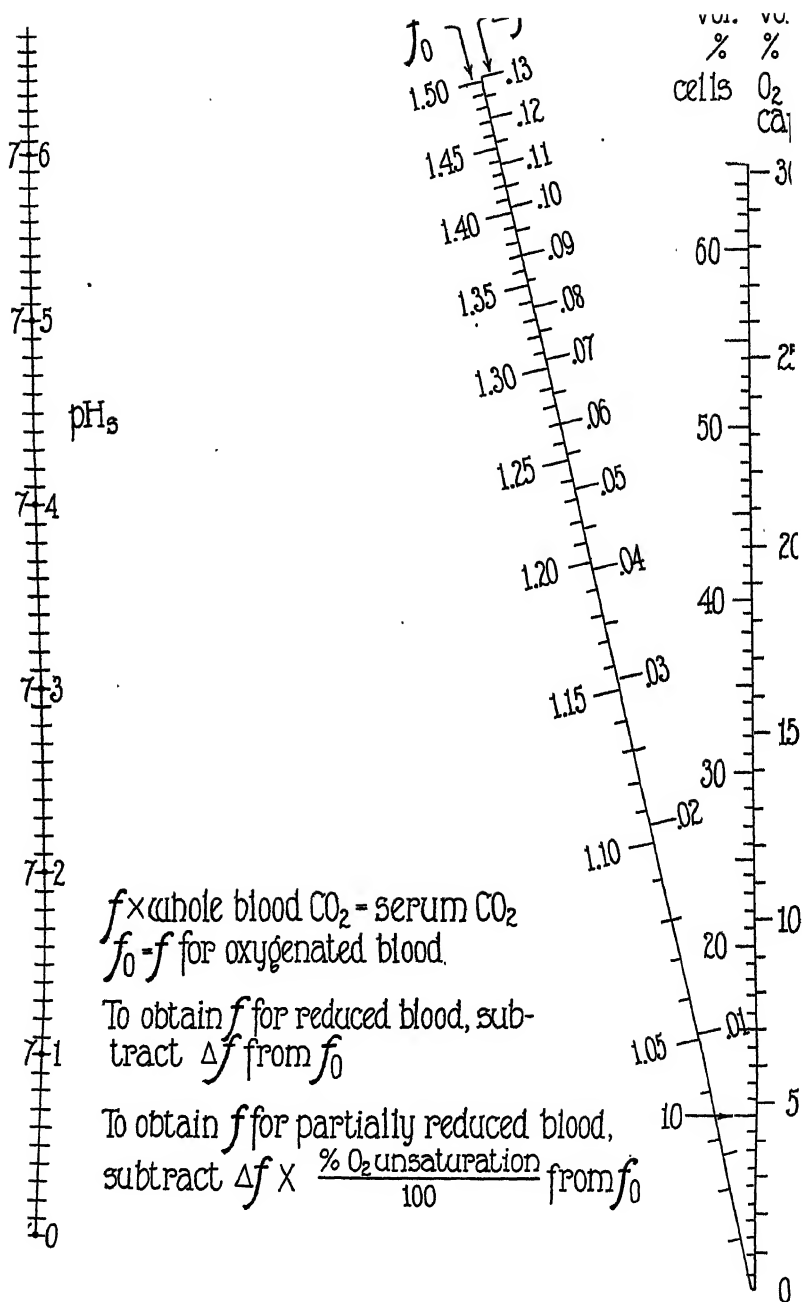


FIG. 3. Line chart for estimating factor f , by which whole blood CO_2 is multiplied to obtain plasma or serum CO_2 .

oxygenated whole blood must be multiplied in order to ascertain the plasma or serum content.

If the hemoglobin in the blood is completely reduced, the intersected value of Δf , indicated by the scale on the right side of the slanting line, is subtracted from the value of f for oxygenated blood.

If, as in venous blood, the hemoglobin is partially reduced, the value subtracted from f is

$$\Delta f \times \frac{\text{per cent O}_2 \text{ unsaturation}}{100}$$

It is essential to know either the oxygen capacity or the cell volume content of the blood in order to make any estimate of the value of f . The latter depends chiefly on the cell content, of which the oxygen capacity serves as a measure. The pH_s and oxygenation exert less pronounced effects on f , and frequently one may use for them assumed values in place of analytically determined figures.

Use of Fig. 3 with Assumed Values for pH_s and Oxygen Unsaturation.—The value of f is not affected much by changes in pH_s within the range ± 0.1 pH. If the blood is drawn from a subject without acidosis or alkalosis, one can assume a pH_s of 7.40 without causing an error, from this source, of over 0.3 volume per cent in the plasma CO_2 calculated.

As is seen from Table I, the change from complete oxygenation to complete reduction of blood lowers the value of f by approximately 4 per cent. Venous blood is usually 40 to 60 per cent reduced. If one assumes that it is 50 per cent reduced the value of f estimated can have a maximum error of 2 per cent, sufficient to cause an error of about 1 volume per cent in the plasma CO_2 estimated in a normal subject. Actually, however, with venous blood drawn under ordinary conditions from the arm, the unsaturation will seldom vary outside the range 25 to 75 per cent; with such blood one can assume 50 per cent unsaturation without involving an error of more than 0.5 volume per cent in the plasma CO_2 calculated.

In arterial blood, except from cyanotic subjects, oxygen unsaturation may be assumed to be 5 per cent.

Construction of Fig. 3.—The following symbols and expressions are used:

[CO₂]_b, [CO₂]_c, and [CO₂]_s represent the CO₂ contents per unit volume in whole blood, cells, and serum respectively.

$f = \text{ratio} \frac{[\text{CO}_2]_s}{[\text{CO}_2]_b}$. Hence f is the desired factor by which whole blood [CO₂]_b is multiplied to find serum [CO₂]_s.

$d = \text{distribution ratio, } [\text{CO}_2]_c : [\text{CO}_2]_s$.

V_s and V_c represent the proportions by volume of serum and cells respectively in the whole blood. $V_s = 1 - V_c$.

We have, either obviously or by definition, the following three equations:

$$(5) \quad [\text{CO}_2]_b = V_s [\text{CO}_2]_s + V_c [\text{CO}_2]_c$$

$$(6) \quad [\text{CO}_2]_c = d [\text{CO}_2]_s$$

$$(7) \quad f = \frac{[\text{CO}_2]_s}{[\text{CO}_2]_b}$$

Combining these three equations we obtain

$$(8) \quad f = \frac{1}{V_s + dV_c}$$

$$(9) \quad d = \frac{\frac{1}{f} - V_s}{V_c}$$

To these equations we add

$$(10) \quad V_c = 2.15 \times (\text{volume per cent O}_2 \text{ capacity})$$

in which the constant, 2.15, is the average of the ratio, cell volume : O₂ capacity, from the data of Peters, Bulger, and Eisenman (1923-24). Equation 10 is only an approximation, and ignores the variations which the above ratio undergoes with changing oxygenation and pH_s. It has, however, been useful.

From Equation 9 it is evident that if one knows the value of d one can calculate the value of f for all possible variations of V_c and V_s .

We have therefore proceeded to ascertain the values of d under varying conditions of oxygenation and pH_s from analyses of human whole blood. Analyses of the necessary type and accuracy are available in papers by Peters, Bulger, and Eisenman (1923-24), Bock, Field, and Adair (1924), Dill *et al.* (1927), Hastings, Neill, Morgan, and Binger (1924), and in a number of unpublished analyses in our laboratory. Values of [CO₂]_c determined by direct analysis of separated human cells are as yet few, but the above sources furnish examples in which [CO₂]_b, [CO₂]_s, and oxygen capacity were determined. From these values we can estimate f by Equation 7, V_c (approximately) by Equation 10, and thence d by Equation 9. The curves representing the mean values of d thus found, at varying pH_s, in

oxygenated and reduced blood, are given in Fig. 4. These served as mother curves for the diagram in Fig. 3.

In calculating the d values for Fig. 4 by means of Equation 9, we have deliberately used, not the V_c by hematocrit, but the V_c values indirectly estimated by Equation 10 from oxygen capacities. This procedure has the advantage that it relates the family of f curves represented in the line chart of Fig. 3 directly to experimentally determined oxygen capacity values, and

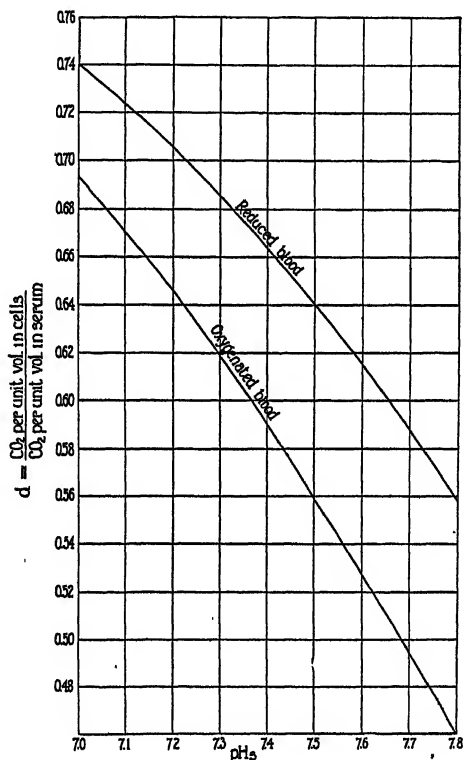


FIG. 4.

makes Fig. 3 applicable with maximum precision with oxygen capacity or hemoglobin content values. As a rule, oxygen capacities are more readily obtained with precision than V_c values by hematocrit. Fig. 3 was accordingly designed primarily for use with the oxygen capacity scale on its right, although it has proved fairly exact when used with the cell volume scale also.

From the d values of Fig. 4, Fig. 3 was constructed as follows: For each of a series of f values for oxygenated blood, three pairs of d and V_c values

were calculated by Equation 9. The three V_o values for each f were arbitrarily chosen at maximum distances apart, and the d values were calculated by Equation 9. For the d values so calculated the corresponding pH_s values were ascertained by interpolation on the curves of Fig. 4. On Fig. 3, where the V_o and pH_s scales were already laid off, three lines were drawn connecting each pair of V_o and pH_s points calculated. Their intersections fell near together, and were located almost exactly on the slanting line of Fig. 3 which bears the f scale. The pH_s and V_o points for a series of f values in reduced blood were similarly determined. The differences between these values for f and those obtained for f in oxygenated blood are expressed on the scale as Δf , which is subtracted from the f for oxygenated blood to obtain f for reduced blood with the same pH_s and V_o .

Comparison with the results of CO₂ determinations on whole blood and plasma in the literature, and in our own records, indicates that with Fig. 3 the plasma CO₂ can be estimated from whole blood CO₂ with an error rarely exceeding 2 volumes per cent, and in the majority of cases under 1 volume per cent. The following tables contain a sufficient number of data to indicate the degree of accuracy with which one may expect to calculate plasma CO₂ from whole blood CO₂ by means of Fig. 3.

NOTES ON DATA IN TABLES.

Table I.—The data on the blood of A. V. B. are from Table IX, Bock, Field, and Adair (1924). All the values for the oxygenated blood of A. V. B. are given in which simultaneous determinations of CO₂ tension, oxygen capacity, and blood and plasma CO₂ contents are recorded in the original. For the reduced bloods no oxygen capacities are recorded in the original paper. The value 18.85 is midway between extreme values 18.2 and 19.5 recorded on other samples of A. V. B.'s bloods. The oxygen unsaturation in the almost completely reduced bloods is estimated by means of the oxygen dissociation curve of A. V. B.'s blood, from the oxygen tensions recorded in the original.

The data on the blood of C. V. C. are obtained by graphic interpolation on the curves of Fig. 2 of Dill, van Caulaert, Hurxthal, Stoddard, Bock, and Henderson (1927). For the interpolation pH_s lines calculated by Equation 3 were drawn, radiating from the origin (*e.g.* see Fig. 1 of Van Slyke, 1921). These lines cut the true serum [CO₂] curves at points indicating the CO₂ tension. The simultaneous whole blood [CO₂] values were obtained by reading the whole blood [CO₂] curves at these CO₂ tensions.

The data on the blood of J. S. and V.-S. are from original observations. The bloods before analysis were equilibrated at 38° with air + CO₂ or N₂ + CO₂ in order to obtain the maximum effects of oxygenation and reduction on f . The analyses were done in duplicate with especial attention to precision. Samples of 3 cc. were used, and a Van Slyke-Neill (1924) apparatus

TABLE I.

Effects of Oxygenation, Reduction, and pH_s on f in Human Blood Equilibrated with Known Gas Mixture.

Data on A.V.B. and C.V.C. from literature. Original data on J.S and V.-S. (see text).

Blood donor.	pH _s calculated from pCO ₂ and [CO ₂] of plasma by Fig. 1.	O ₂ capacity.	O ₂ unsaturation.	[CO ₂] _s in serum or plasma.	[CO ₂] _b in whole blood.	$f = \frac{[\text{CO}_2]_s}{[\text{CO}_2]_b}$ Found.	f from Fig. 3. Calculated.	Difference f calculated - f found.
		vol. per cent	per cent	vol. per cent	vol. per cent			
A. V. B., oxalated blood. From Bock, Field, Adair (1924).	7.20	19.5	0	75.1	64.2	1.169	1.175	+0.006
	7.20	19.5	0	75.0	63.5	1.182	1.175	-0.007
	7.22	19.5	0	73.7	62.7	1.175	1.177	+0.002
	7.22	19.5	0	73.5	62.4	1.178	1.177	-0.001
	7.55	19.5	0	49.5	39.5	1.255	1.237	-0.018
	7.55	19.5	0	48.7	39.7	1.227	1.237	+0.010
	7.24	(18.85)	98	78.3	68.9	1.136	1.138	+0.002
	7.24	(18.85)	98	77.2	68.5	1.127	1.138	+0.011
	7.28	(18.85)	98	74.4	65.3	1.140	1.144	+0.004
	7.58	(18.85)	94	54.5	46.4	1.175	1.188	+0.013
	7.60	(18.85)	97	52.6	44.9	1.172	1.189	+0.017
	7.61	(18.85)	97	51.9	44.4	1.169	1.191	+0.022
C. V. C., defibrinated blood. From Dill, van Cault, et al., (1927).	7.20	20.0	0	76.0	64.5	1.178	1.180	-0.002
	7.20	20.0	100	82.8	72.2	1.147	1.144	-0.003
	7.30	20.0	0	68.0	57.0	1.192	1.194	-0.002
	7.30	20.0	100	75.5	65.6	1.150	1.155	+0.005
	7.40	20.0	0	61.0	50.8	1.200	1.212	+0.012
	7.40	20.0	100	68.8	59.2	1.162	1.168	+0.006
	7.50	20.0	0	54.0	44.2	1.222	1.235	+0.013
	7.50	20.0	100	62.0	52.3	1.185	1.186	+0.001
	7.60	20.0	0	48.0	38.0	1.262	1.258	-0.004
	7.60	20.0	100	54.0	45.0	1.200	1.202	+0.002
J. S. defibrinated.	7.36	22.2	0	59.72	47.95	1.245	1.233	-0.012
	7.39	22.2	89	65.25	54.36	1.201	1.191	-0.010
V.-S. oxalated.	7.33	17.8	0	57.50	48.86	1.177	1.174	-0.003
	7.34	17.8	96	64.80	56.83	1.140	1.140	±0.000

was employed with a chamber calibrated at 1.0, 4.0, and 100.0 cc., instead of the usual 0.5, 2.0, and 50 cc. The CO₂ and O₂ contents of the whole blood were determined in the same 3 cc. samples; the CO₂ pressures were read at 4.0 cc. volume, the O₂ pressures at 1.0 cc. The "oxygen unsaturation" values are calculated from the oxygen contents thus determined.

Table II.—These results are from venous blood, analyzed in this laboratory as drawn, in the usual Van Slyke-Neill apparatus. 2 cc. samples were used for oxygen content and oxygen capacity, 1 cc. for CO₂ content. The bloods were centrifugated with precautions described in Paper I of this series (Austin *et al.*, 1922) to prevent loss of CO₂, and the plasma CO₂ was determined in 1 cc. samples.

TABLE II.

Comparison of Observed and Calculated f Values in Normal Venous Human Blood, Analyzed As Drawn (Oxalated).

Original data.

Blood donor.	pH, c = colori- metric. s = electro- metric.	O ₂ capacity.	O ₂ unsatu- ration.	[CO ₂] _p in plasma.	[CO ₂] _b in whole blood.	$f =$ $\frac{[\text{CO}_2]_p}{[\text{CO}_2]_b}$ Found.	f from Fig. 3. Calcu- lated.	Differ- ence f calcu- lated - f found.
		vol. per cent	per cent	vol. per cent	vol. per cent			
A. B. H.	7.41 e	19.5	58	68.8	58.3	1.180	1.182	+0.002
J. N.	7.40 e	18.4	63	74.4	63.5	1.171	1.168	-0.003
R. B.	7.37 c	17.6	53	69.7	61.7	1.129	1.158	+0.029
C. B.	7.47 c	17.6	10	67.3	56.2	1.196	1.188	-0.008
C. L.	7.43 e	19.8	28	63.4	52.6	1.205	1.204	-0.001
T. S.	7.41 c	16.5	62	62.1	53.0	1.171	1.150	-0.021
J. S.	7.41 c	20.2	46	74.2	61.4	1.208	1.196	-0.012

Table III.—From Hastings, Neill, Morgan, and Binger (1924). The technique was that described for Table II.

Table IV.—The data are partly original, partly from Peters, Bulger, and Eisenman's (1923-24) values on oxygenated blood equilibrated with air containing CO₂ at 40 mm. pressure. Of their determinations, which total over 100, only the first twenty are reproduced: these illustrate the variations sufficiently. The pH_s values given here for this group of bloods are 0.03 greater than those tabulated in the original paper: the change is to correspond with the higher pK' value published by Hastings, Sendroy, and Van Slyke (1928), which is the basis of calculation in the present paper.

The results in Table IV show that, although Fig. 3 was designed primarily for estimations of f based on oxygen capacities, the cell

volume scale can be used instead of the O_2 capacity scale without serious increase in the error of the estimation.

The data on horse blood also show that the f values for it do not differ greatly from those for human blood.

The calculated values of f from the data of Peters, Bulger, and Eisenman show from the observed values considerably greater mean and maximum deviations than those in any of the other groups of analysis tabulated in this paper. The deviations are about the same, whether the calculated f values are from Peters' chart or from ours. The greater variability of these bloods is

TABLE III.

Comparison of Observed and Calculated f Values in Arterial Blood from Pneumonia Patients, Analyzed As Drawn (Oxalated).

Data of Hastings, Neill, Morgan, and Binger (1924).

No. in original paper.	pH _s electro-metric.	O ₂ capacity.	O ₂ unsaturation.	[CO ₂] _p in plasma.	[CO ₂] _b in whole blood.	$f = \frac{[CO_2]_p}{[CO_2]_b}$ Found.	f from Fig. 3. Calculated.	Difference f calculated — f found.
		vol. per cent	per cent	vol. per cent	vol. per cent			
18	7.44	14.1	5.6	58.0	51.6	1.123	1.142	+0.021
19	7.43	16.4	3.0	61.7	52.8	1.168	1.171	+0.003
20	7.42	21.3	8.4	59.0	48.1	1.226	1.231	+0.005
21	7.48	19.2	9.8	62.0	51.4	1.206	1.216	+0.010
25	7.47	18.4	3.8	52.4	43.6	1.201	1.201	±0.000
29	7.51	16.5	16.3	55.1	45.7	1.205	1.181	-0.024

presumably due to the fact that they are from a miscellaneous group of hospital patients, many of whom were obviously in very pathological condition. Probably these data present differences between estimated and observed f values as great as one is likely to encounter. The maximum difference of 0.05 in f would involve an error of about 2.5 volumes per cent in plasma CO_2 estimated from whole blood CO_2 by multiplication with the f value from Fig. 3. In the majority of blood analyses presented in the accompanying tables the difference between observed and calculated f is not over 0.02, and the corresponding error in estimating plasma or serum CO_2 from whole blood CO_2 is not over 1 volume per cent.

TABLE IV.
Comparison of Observed f Values with Values Calculated from Oxygen Capacity and Cell Volume Respectively.

Material.	pH _s by Hasselbalch equation (Fig. 1).	O ₂ capacity.	Cell volume by hematocrit.	Ratio cell volume O ₂ capacity.	O ₂ unsaturation.	[CO ₂] _p in plasma (or serum).	[CO ₂] _b in whole blood.	Value of $f = [\text{CO}_2]_p : [\text{CO}_2]_b$.			
								Observed.	O ₂ capacity.	Calculated by Fig. 3 from:	Calculated by Peters chart from O ₂ capacity.
		vol. per cent	vol. per cent		per cent	vol. per cent	vol. per cent			Cell volume.	
Defibrinated blood of J. S.	7.36	22.2	48.4	2.18	0	59.72	47.95	1.245	1.233	1.238	
	7.39	22.2	49.6	2.23	89	65.25	54.36	1.201	1.186	1.193	
Oxalated blood of V.-S.	7.33	17.8	38.3	2.15	0	57.50	48.86	1.177	1.174	1.175	
	7.34	17.8	39.2	2.20	96	64.80	56.83	1.140	1.135	1.142	
Defibrinated horse blood.	6.96	17.8	37.2	2.09	5	82.60	73.20	1.128	1.130	1.128	
	7.00	17.8	38.4	2.16	95	85.30	77.76	1.096	1.098	1.106	
" "	7.43	16.8	34.0	2.02	0	59.85	50.69	1.180	1.177	1.165	
	7.46	16.8	35.0	2.08	89	64.35	56.12	1.147	1.149	1.142	
" "	7.69	17.8	35.1	1.97	0	37.00	30.06	1.231	1.232	1.205	
	7.66	17.8	36.1	2.03	77	40.72	34.72	1.173	1.196	1.184	

[illegible]

SUMMARY.

In Fig. 1 is presented a line chart of the d'Ocagne-Henderson type for use in graphic calculations by the Henderson-Hasselbalch equation. The values of the constants, α_{CO_2} , and pK' , for human serum, arrived at in recent papers of this series, are applied. Of the twelve calculations that can be made on Fig. 1 by combining three at a time the four variables, $[\text{CO}_2]_s$, $[\text{BHCO}_3]_s$, pH_s , and CO_2 tension, ten calculations are sufficiently exact to be of practical use.

In Fig. 3 is presented a line chart for graphic estimation of the CO_2 content of plasma or serum from the CO_2 content observed in whole blood of any hemoglobin or cell content, pH_s , and degree of oxygenation.

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THE BEHAVIOR OF CASEIN IN PARTIAL SOLUTION IN CALCIUM HYDROXIDE.

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INTRODUCTION.

The combination of casein with calcium hydroxide has been studied by a number of investigators (1). The majority analyzed the compound of this protein and $\text{Ca}(\text{OH})_2$ for the inorganic element; the results thus obtained were rather inconsistent. The cause of this inconsistency may become apparent through our experiments.

Van Slyke and Bosworth (2) in their classical study of casein found that 2.25×10^{-4} equivalents of $\text{Ca}(\text{OH})_2$ are just sufficient to hold 1 gm. of casein in solution. This value was found to be twice the corresponding value for monovalent base.

Schryver (3) equilibrated casein with lime water and upon separating the undissolved part found that "the solvent capacity of NaOH approximates to that of an equimolar (not equinormal) solution of $\text{Ca}(\text{OH})_2$." Schryver applied the criteria of solubility to the study of the denaturation of casein. According to this investigator there exist at least two modifications of casein: One which he named "metacaseinogen," is produced from casein by heat and by long standing with acetic acid at a concentration of 1:10,000; the other, paracasein, is produced by rennin or pepsin. Metacaseinogen may be converted back to casein, while paracasein cannot. Both of these derivatives do not differ from casein in their N:P ratio.

Kondo (4), and Linderstrøm-Lang and Kodama (5) carried out an extensive investigation upon the solubility of casein in hydrochloric acid. Although these authors were using a different solvent from us, their conclusions are of such importance to the chemistry of casein that we shall dwell upon them in some detail.

Kondo (4) dissolved casein (prepared by the method of Hammersten) in varied amounts of HCl . "Measurements of the solubility of casein in HCl revealed the peculiar fact that the solubility decreases with the same concentration of HCl to begin with—as the quantity of precipitate in

contact with the solution is increased." Linderstrøm-Lang and Kodama (5) carried these measurements further upon carefully purified casein preparations, which were freed from fats by ether, but were not treated by alcohol. The investigation of the solubility of casein in HCl was carried out in the presence of varied amounts of NaCl, in such a manner that the concentration of Cl, in any given set of experiments, was constant.

The evidence brought about by the study of the different fractions of the preparation of casein, led these authors to the following conclusions: "There can hardly be any doubt that casein, as liberated at the isoelectric point, is a mixture of two or more components united in the precipitate by the forces assumed by Sørensen. On treating the precipitate with HCl it is very difficult to separate the components one from another, but something can be extracted after long subjection to the process, chiefly the more easily soluble portions. . . . The chemical differences of the component groups may be characterized partly by their different behavior in regard to colloidal precipitation with calcium salt, partly by the proportion between phosphorus and nitrogen, as previously utilized by Sørensen for characterization of the globulins."

Gortner (6) criticized the conclusion of Linderstrøm-Lang and Kodama in regard to their belief that casein is a mixture. He pointed out that the casein used by these investigators was not purified by alcohol: there remained an alcohol-soluble protein identified by Osborne and Wakeman (7). The chemical difference attributed to casein by Linderstrøm-Lang and Kodama, according to Gortner, may be due to the presence of this foreign protein.

Greenberg and Schmidt (8) studied the transport numbers of the compound of casein with the alkali earth elements. They found that: "The values for the transport numbers of casein in solutions of the alkali earth elements are abnormally high as compared with the values which were obtained for casein in solutions of the alkali metals. The abnormal values are probably due to the fact that part of the alkali earth element is bound by the casein ion in a non-ionic form, producing a complex which carries a certain amount of the element in a direction opposite to that of the cation." This phenomenon was found to be similar in some respects, to absorption.

It is difficult to derive any clear notion about the behavior of casein in the problems dwelt upon in this section. As far as we can see, we may conclude that: (a) Casein kept at the isoelectric point does not retain its solubility toward $\text{Ca}(\text{OH})_2$ indefinitely (Schryver). This was confirmed by Linderstrøm-Lang and Kodama (5) in regard to HCl. According to Schryver (3) this change in solubility does not necessarily involve a change in the N:P ratio. (b) From (a) we may conclude that in some cases the change in the solubility of casein does not depend upon its elementary composition. The two properties of casein may vary independ-

ently. (c) The solubility of casein in a given amount of HCl is a function of the amount of protein in the system. The criticism advanced by Gortner may not apply to this case, since a casein preparation (Kondo (4)) prepared by the method of Hammersten (which involves a purification with alcohol) displayed the same peculiar property as the preparation which was not treated by alcohol. (d) However, the criticism advanced by Gortner holds true concerning the composition of casein as gathered by chemical analysis.

EXPERIMENTAL.

Preparation of Casein.

Casein was obtained from cow's milk, two methods of preparation being used.

First Method.—Commercial certified milk was used as a source of the protein. The casein was precipitated by HCl, washed at the isoelectric point with distilled water, and redissolved with NaOH until the reaction was pH 6.5. Then the casein solution was passed through a Sharples centrifuge and paper pulp filter, precipitated with HCl, and again washed at the isoelectric point. This operation was repeated once or twice. The casein was then kept at 5° under toluene. These procedures have already been described in detail (9). Casein Preparations XXII, XXV, and XXVII were prepared by this method.

Second Method.—To 4 liters of skimmed milk from a single cow, delivered at the laboratory within 2 hours of milking, 30 gm. of sodium oxalate were added. The milk was stirred for $\frac{1}{2}$ hour and then left undisturbed overnight at 5°. In the morning the milk was syphoned off from the precipitate of the insoluble calcium salts and passed through a Sharples centrifuge. The casein was then precipitated and purified as by the first method. Casein Preparation XXIX was prepared by this method.

As far as the solubility of these preparations is concerned, they all behaved very much alike (Fig. 1). The variation between individual preparations as regards their solubility in $\text{Ca}(\text{OH})_2$ is not much greater than the one observed in the solubility of this protein in NaOH (10). The properties of casein do not depend upon whether the casein was precipitated from the milk of one cow or the mixture of many. The two methods yielded similar casein preparations.

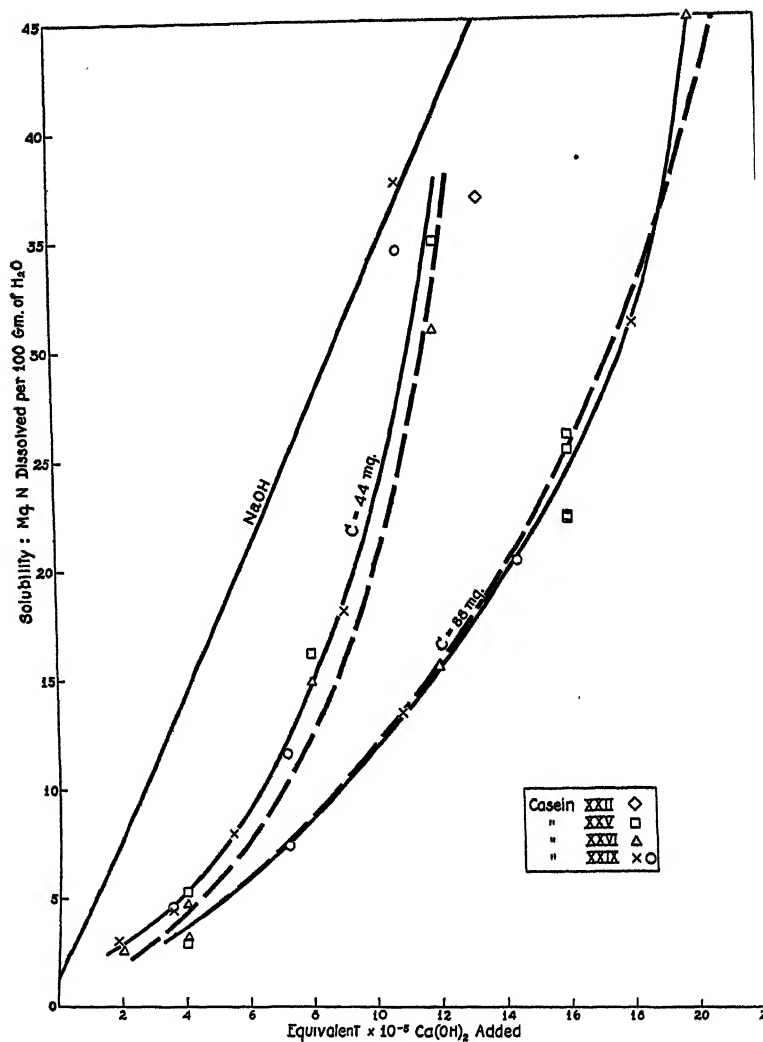


FIG. 1. The solubility of casein in $\text{Ca}(\text{OH})_2$ at 25° . Solid lines show experimental curves; broken lines calculated with the help of the equation

$$S = \frac{0.850}{\frac{1}{\text{Ca}(\text{OH})_2} - \frac{2.55}{C}}$$

The straight line is the solubility of casein in NaOH (10) at the same temperature.

Measurements of Solubility.

The technique used has been already described elsewhere (9). It consisted in bringing into intimate contact a suspension of isoelectric casein of known concentration with $\text{Ca}(\text{OH})_2$ solutions. After equilibrium conditions were established the undissolved casein was filtered off through a No. 42 Whatman filter paper, and the amount of protein dissolved was determined by analysis for nitrogen, by the Kjeldahl method. These analyses were made in triplicate. Toluene was used as an antiseptic.

The equilibrium conditions were brought about by the continuous shaking of the flasks containing casein and $\text{Ca}(\text{OH})_2$ in an efficient

TABLE I.
Test for Equilibrium Conditions.

Experiment 6, Casein Preparation XXV.

Amount of $\text{Ca}(\text{OH})_2$ added, 16.05×10^{-5} equivalents.

" " casein in system, 88 mg. of N.

Temperature $25.0 \pm 0.2^\circ$.

Period of shaking.	Solubility. Mg. N dissolved per 100 gm. H_2O .
<i>hrs.</i>	
6	22.2
12	22.4
24	(24.4)
36	22.3

shaking machine. The rate of shaking may be described as being sufficient to prevent any casein particles from settling on the bottom of the flasks. A test for equilibrium conditions is given in Table I.

Table I indicates that a continuous shaking for 6 hours is sufficient to bring about a state of rest. As a precaution, however, we shook our suspensions never less than 40 hours.

For the study of their solubility in $\text{Ca}(\text{OH})_2$, casein preparations cannot be used indefinitely; from 3 to 4 weeks after their final purification they become less soluble. In this we confirm the observations of Schryver (3) upon the properties of casein.

Determination of Amount of Base Present in Solution.

Besides knowing how much casein has been dissolved, it is extremely interesting to know how much of the originally added base

is present in solution. For the determination of the base recovered from the solution, we used a method very similar to that

TABLE II.

Solubility and Amount of Base in Solution in Systems Composed of Casein and $\text{Ca}(\text{OH})_2$.

Temperature $25 \pm 0.5^\circ$.

Experiment No.	Casein preparation No.	Equivalents $\times 10^{-4} \text{ Ca}(\text{OH})_2$ added.	Solubility. Mg. N dissolved per 100 gm. H_2O .	Amount of base in solution. Equivalents $\times 10^{-4}$ per 100 gm. H_2O .
Total amount of casein = 44 mg. N in 100 gm. H_2O .				
69	XXIX	1.81	3.00	1.72
13	XXVI	2.00	2.5	
68	XXIX	3.62	4.60	2.50
2	XXV	4.01	5.3	
13	XXVI	4.01	4.7	
69	XXIX	5.43	8.00	4.25
68	"	7.24	11.65	5.60
8	XXV	8.03	16.2	
13	XXVI	8.03	14.9	
69	XXIX	9.05	18.15	7.75
68	"	10.9	34.5	
69	"	10.9	36.6	
2	XXV	12.04	34.9	
13	XXVI	12.04	30.8	
11	XXII	13.4	36.8	
Total amount of casein = 88 mg. N in 100 gm. H_2O .				
69	XXIX	3.62	4.50	1.95
8	XXV	4.01	2.9	
13	XXVI	4.01	3.1	
68	XXIX	7.24	7.40	3.25
69	"	10.9	13.45	6.30
13	XXVI	12.0	15.5	
68	XXIX	14.5	20.35	9.50
8	XXV	16.05	25.0	
69	XXIX	18.15	31.1	14.10
13	XXVI	20.05	45.4	

described by Linderström-Lang and Kodama (5). Aliquot parts of the solution of casein, left from the solubility measurements, were

diluted with twice their volume of distilled water. The solutions were then titrated with 0.01 and 0.001 N HCl to a standard containing casein suspension at pH 4.7. Methyl red was used as the indicator.

This method yielded reproducible results except when large amounts of casein were dissolved. Under these circumstances,

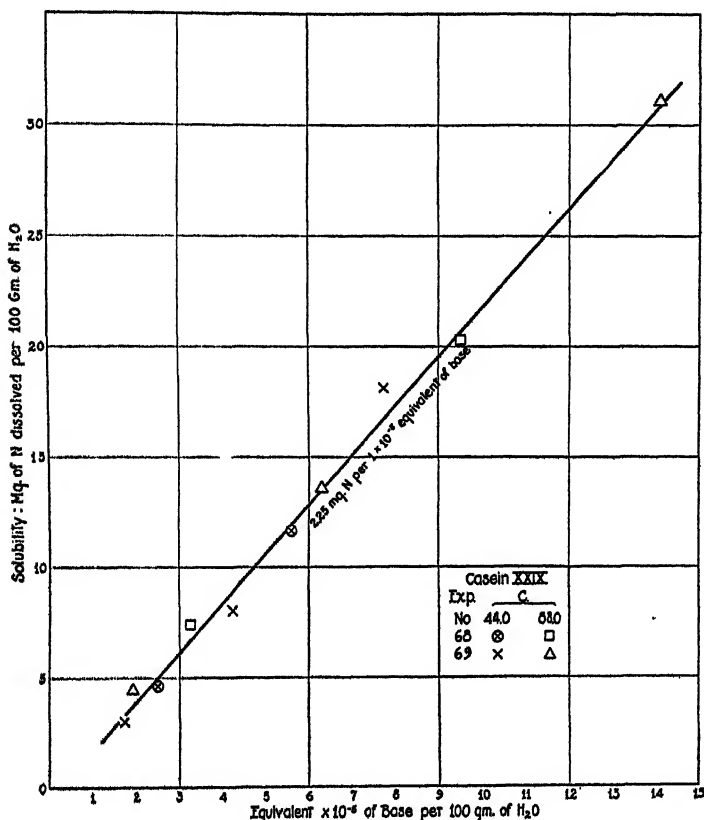


FIG. 2. The amount of $\text{Ca}(\text{OH})_2$ bound by casein in solution.

apparently, there was a time factor. We have therefore omitted from our tables determinations of base in solution in systems containing large amounts of casein.

The results of our determinations of the solubility and amounts

of base present in solution, in suspensions containing casein and $\text{Ca}(\text{OH})_2$, are given in Table II.

DISCUSSION.

From inspection of Fig. 1 the following seems to be apparent: Equal amounts of $\text{Ca}(\text{OH})_2$ added, do not carry equal amounts of casein into solution throughout the solubility range; *i.e.*, first casein dissolves at a slower rate, then a small increase in the $\text{Ca}(\text{OH})_2$ added carries considerable amounts of casein into solution. Outside these considerations the solubility of casein depends upon the amount of casein in the system. This phenomenon is very similar to the one described by Kondo (4) and Linderstrøm-Lang and Kodama (5) in their study of the solubility of casein in HCl .

The relationship between the amount of base in solution and the amount of casein in the same solution (Fig. 2), is surprisingly simple. Throughout the range investigated, an equal increase in the amount of casein dissolved is associated with an equal increase in the amount of base recovered from the same solution. 1×10^{-5} equivalents of $\text{Ca}(\text{OH})_2$ is bound to 2.25 mg. of casein N.

Throughout this paper, we will use the following symbols: (1) S for the amount of casein dissolved. S is expressed in mg. of casein N per 100 gm. of water. (2) P for the fraction of casein left undissolved; *i.e.*, the solid phase. P is similarly expressed as mg. of casein N per 100 gm. of water. (3) C for the total amount of casein in the system equal to $S + P$. Its numerical value is expressed as in (1) and (2). (4) $\text{Ca}(\text{OH})_{2S}$ for the equivalents of $\text{Ca}(\text{OH})_2$ recovered from the solution, multiplied by 10^5 , in order to avoid the continuous use of the proportionality constant between the mg. of N and the equivalents of base added. $\text{Ca}(\text{OH})_{2S}$ is similarly expressed per 100 gm. of H_2O . (5) $\text{Ca}(\text{OH})_{2P}$ for $\text{Ca}(\text{OH})_2$ in precipitate, expressed as in (4). (6) $\text{Ca}(\text{OH})_2$ equals $\text{Ca}(\text{OH})_{2S} + \text{Ca}(\text{OH})_{2P}$. This is the amount of the base that was added to the system.

S , C , $\text{Ca}(\text{OH})_{2S}$, and $\text{Ca}(\text{OH})_2$ were measured directly. P and $\text{Ca}(\text{OH})_{2P}$ may be calculated with the help of the following equations:

$$P = C - S \quad (1)$$

$$\text{Ca}(\text{OH})_{2P} = \text{Ca}(\text{OH})_2 - \text{Ca}(\text{OH})_{2S} \quad (2)$$

These suppositions do not involve any assumptions except that there was no considerable change in the redistribution of water between the solution of casein and the precipitate, as compared with the same system to which no $\text{Ca}(\text{OH})_2$ was added.

The description of the system casein- $\text{Ca}(\text{OH})_2$ which we gave at the beginning of this section, may be summarized in the following way: The solubility is intimately related to the amount of casein forming the solid phase. This observation leads us to the supposition that the variables involved in the system of casein and $\text{Ca}(\text{OH})_2$ may be interrelated by assuming that the amount of casein in solution corresponds to two solubilities or two modes of combination of casein with $\text{Ca}(\text{OH})_2$: one which increases with the amount of casein in solution, and the other which increases proportionally to the amount of casein in the solid phase. This supposition may be described by the following equation:

$$S = K_1 \text{Ca}(\text{OH})_2 \frac{S}{C} + K_2 \text{Ca}(\text{OH})_2 \frac{P}{C} \quad (3)$$

where $P = C - S$ and K_1 and K_2 are the proportionality constants describing the rate at which the two combinations take place in terms of equation (3). $\frac{S}{C} + \frac{P}{C} = 1$. When practically all of the

casein is in solution the $K_2 \text{Ca}(\text{OH})_2 \frac{P}{C}$ becomes vanishingly small. Conversely, when little solution of casein has taken place, the solubility is practically dependent upon the term $K_2 \text{Ca}(\text{OH})_2 \frac{P}{C}$ and is nearly independent of the amount of casein in the system since the ratio $\frac{P}{C}$ approaches unity. Furthermore $\text{Ca}(\text{OH})_2 \frac{S}{C} + \text{Ca}(\text{OH})_2 \frac{P}{C} = \text{Ca}(\text{OH})_2$; the fractions $\frac{S}{C}$ and $\frac{P}{C}$, therefore govern the distribution of $\text{Ca}(\text{OH})_2$ between the two modes of combination assumed to take place.

In the form (Equation 3) the equation can hardly be of use, since it contains both sides of the term S . Solving for S we obtain

$$S = \frac{K_2}{\frac{1}{\text{Ca}(\text{OH})_2} - \frac{K_1 - K_2}{C}} \quad (4)$$

With the help of this equation and $K_1 = 3.40$, $K_2 = 0.850$, the values of S were calculated. They are reproduced in Fig. 1 (dash line). They seem, on the whole, to agree with the values obtained experimentally.¹

TABLE III.
Significance of Constants Used.

Constant.	Value mg. N per 1×10^{-3} equiva- lents of base.	Value gm. casein per 1 equiva- lent of base.	Physicochemical significance, as previously identified.	Obtained by:
K_1	3.40	2180	Equivalent combining weight from solubility measurements, electrochemical equivalent of casein with monovalent base, and from "formol titration."	Cohn and Hendry (10). Greenberg and Schmidt (11). Long (12). Robertson (13, 14). Carpenter (15).
K_2	0.850	545	Maximum base-combining capacity of all acid groups of casein with monovalent base.	Cohn and Berggren (16). Robertson (17).
K_3	2.25	1440	Equivalent combining weight of paracasein from solubility measurements with NaOH.	Pertzoff (18).

Outside this relation we have one describing the interdependence of the casein in solution and the amount of $\text{Ca}(\text{OH})_2$ bound by it. The equation as we have already mentioned is

$$S = K_3 \text{Ca}(\text{OH})_2 S \quad (5)$$

with the proportionality constant $K_3 = 2.25$. This follows directly from the straight line relationship of Fig. 2 and holds true,

¹ The calculated values should be corrected for the origins of the solubility lines. In the case of the solubility of casein in NaOH at 25° the line originates at 1.3 mg. of N per 100 cc. of H_2O (10). Although such a correction may improve our calculated values, we have anticipated its use, believing in our case its value cannot be determined without certain assumptions.

as far as we have carried out our investigations, until about one-third of the casein has dissolved.

The whole interest of this solution lies in the fact that the constants used are not empirical in their nature, but may be identified with the physicochemical constants already derived for casein. $K_1 = 3.40$. If we convert this value into gm. of casein per 1 equivalent of base (using the nitrogen factor 6.41), we obtain 2180 gm. Similarly K_2 yields 545 gm. and K_3 , 1440 gm. In Table III are compiled the results of this calculation as well as the physicochemical nature of the constants thus derived.

Constant K_1 is equal to the rate at which casein dissolves with monovalent base. K_2 is proportional to all the acid groups available in the casein molecule. The ratio of casein in solution to the amount of base in the same solution is equal to the solubility of a derivative of casein, paracasein, in NaOH.

These conclusions seem significant: the very fact that by the use of our equation we fell upon constants describing well known properties of casein, is itself the best justification for the existence of this relation.

Solubility of Casein in NaOH at 5°.

Could the properties of casein observed in the system composed of this protein and $\text{Ca}(\text{OH})_2$ be identified in any other system containing this protein and a base? We are fortunate in being able to answer in the affirmative. The solubility of casein in NaOH at 5° may be characterized as due to the same cause which governs the solubility of this protein in $\text{Ca}(\text{OH})_2$.

The solubility of casein in NaOH at 25° was extensively studied by Cohn and Hendry (10). The same system at 5° was studied by us in regard to the solubility of casein in small amounts of NaOH (14). In terms of the equation $S = K_1\text{Ca}(\text{OH})_2 \frac{S}{C} + K_2\text{Ca}(\text{OH})_2 \frac{P}{C}$ we deal with a limiting case where S is almost solely determined by the term $K_2\text{Ca}(\text{OH})_2 \frac{P}{S}$ and since little solubility has taken place the ratio $\frac{P}{C}$ is nearly equal to unity. In other words, the solubility is independent of the amount of casein in the system, which was confirmed by our experiments.

However, when larger amounts of NaOH were added to casein, the situation became like the one described for the system casein- $\text{Ca}(\text{OH})_2$. This necessitated the use of casein suspensions of constant concentration and the results of such measurements made upon a system composed of 100 mg. of casein N in 100 gm. of H_2O , is given in Table IV and is graphically reproduced in Fig. 3 together with the solubility of casein in small amounts of NaOH, borrowed from our previous experiments (14). The values of

TABLE IV.
Solubility of Casein in NaOH at 5°.
Casein Preparation XXVII.

Total amount of casein, 100 mg. of N in 100 gm. of H_2O .

Experiment No.	Time for equilibrium.	Mols NaOH $\times 10^{-3}$ added.	Solubility. Mg. N per 100 gm. H_2O .
	<i>hrs.</i>		
52	18	8.00	17.25
47	17	10.00	23.9
51	21	10.00	21.1
47	17	15.00	39.7
51	21	15.00	39.9
51	21	20.00	53.2
42	42	20.00	51.8
47	17	25.00	76.5
51	21	25.00	75.6
47	17	30.00	95.9
42	42	30.00	95.7

Table IV were obtained by following the procedure described in an early section of this paper.

With the help of the equation:

$$S = \frac{K_4}{\frac{1}{\text{NaOH}} - \frac{K_1 - K_4}{C}}$$

(assigning the values $K_1 = 3.40$ and $K_4 = 2.00$) we calculated the solid line of Fig. 3. It agrees well with the experimental points and undoubtedly describes the phenomenon in question.

Is it possible to identify the physicochemical nature of the constants used? $K_1 = 3.40$. In terms of gm. of casein per 1

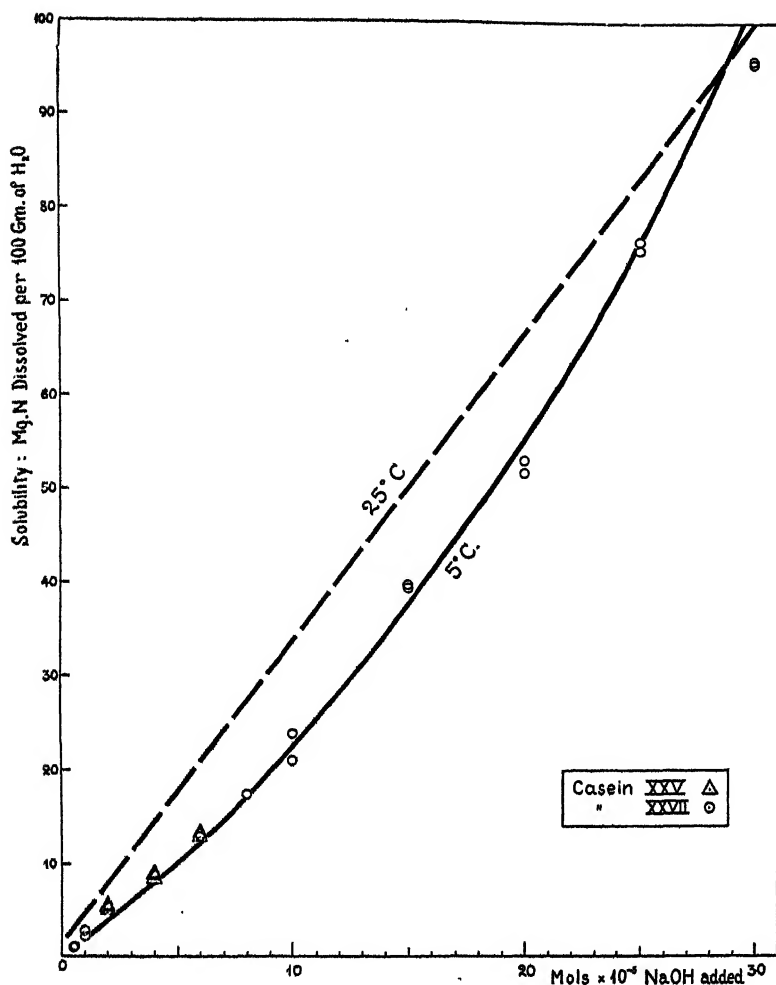


FIG. 3. The solubility of casein in NaOH at 5°. Solid line calculated with the help of the equation:

$$S = \frac{2.00}{\frac{1}{\text{NaOH}} - \frac{1.40}{C}}$$

Broken line represents the solubility of casein in the same base at 25° (10).

equivalent of base, it is equal to 2180. This is the same constant which we used for the system of casein- $\text{Ca}(\text{OH})_2$ and is equal to the rate at which casein dissolves at 25° . The constant K_4 is a new constant and at present cannot be identified with any other constants of casein, perhaps for the reason that the behavior of this protein, at low temperature, has been little studied.

Whatever may be the physicochemical significance of the constant K_4 , it is apparently the only unknown in the equation

$$S = \frac{K_4}{\frac{1}{\text{NaOH}} - \frac{K_1 - K_4}{C}}$$

All other values are either experimentally determined, or represent characteristic values of casein determined elsewhere. It is of interest, that in the case of the solubility of casein in NaOH at 5° , a similar relation holds true, as in the case of the solubility of this protein in $\text{Ca}(\text{OH})_2$. These two behaviors have a common cause—a curious physicochemical mechanism—inherent in the complicated chemical structure of casein.

SUMMARY.

1. The solubility and the amount of base in solution in the system composed of casein and $\text{Ca}(\text{OH})_2$ were studied.

2. It was found that in the system casein- $\text{Ca}(\text{OH})_2$ the solubility as well as the amount of base in solution, depended not only upon the amount of $\text{Ca}(\text{OH})_2$ added, but also upon the concentration of casein.

3. The relation:

$$S = \frac{K_2}{\frac{1}{\text{Ca}(\text{OH})_2} - \frac{K_1 - K_2}{C}}$$

was found to describe the experimental results. In this equation S is the amount of casein dissolved, $\text{Ca}(\text{OH})_2$ the base added, and C the total amount of casein in the system.

4. The relation between the amount of casein dissolved and $\text{Ca}(\text{OH})_2$ bound by it was found to be a linear one, described by the equation $S = K_3 \text{Ca}(\text{OH})_{2S}$.

5. The values of K_1 , K_2 , and K_3 in terms of gm. of casein per 1 equivalent of base, were found to be 2180, 545, and 1440 respectively. These values were previously identified as (1) the equivalent combining weight of casein with monovalent base, (2) the maximum base-combining capacity of casein, and (3) as the equivalent combining weight of a derivative of casein, paracasein.

6. We may therefore conclude that it is possible to interpret the behavior of casein toward $\text{Ca}(\text{OH})_2$ in terms of physicochemical constants, characteristic of casein and previously derived by various investigators.

7. The solubility of casein in large amounts of NaOH was studied at 5° and found to possess the same properties as the system of casein and $\text{Ca}(\text{OH})_2$ at 25° .

8. The equation derived for casein- $\text{Ca}(\text{OH})_2$ was found to hold true for the system of casein- NaOH at 5° .

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